

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.: **WARF-0002**

Inventors: **Laughon, Allen S.**

Serial No.: **09/810,385**

Filing Date: **March 16, 2001**

Examiner: **Harris, Alana M.**

Customer No.: **26259**

Group Art Unit: **1643**

Confirmation No.: **8778**

Title: **Compositions and Methods for Negative Regulation of TGF- β Pathways**

Electronically Submitted via EFS-Web
Date: January 15, 2008

I hereby certify that this paper is being electronically submitted on the date indicated above to the Commissioner for Patents, U.S. Patent & Trademark Office (AF).

By Janine Massey Licata
Typed Name: **Janine Massey Licata, Reg. No. 32,257**

Commissioner for Patents
U.S. Patent & Trademark Office
(AF)

APPEAL BRIEF

TABLE OF CONTENTS

I.	Real Party in Interest.	1
II.	Related Appeals and Interferences.	1
III.	Status of Claims	1
IV.	Status of Amendments	1
V.	Summary of Claimed Subject Matter	3
VI.	Grounds of Rejection to be Reviewed on Appeal.	5
VII.	Arguments	5
	Issue: Whether the Claimed Subject Matter Lacks Written Description and is Indefinite	
	A. The Rejection of Claims 9-12 Under 35 U.S.C. §112, First Paragraph Should Be Withdrawn	5
	B. The Rejection of Claims 9-12 Under 35 U.S.C. §112, Second Paragraph Should Be Withdrawn.	8
VIII.	Claims Appendices	10
	Appendix A - Pending Claims	10
	Appendix B - Proposed Claim Amendments (offered in Response to Advisory Action but not entered per Advisory Action Before Filing of an Appeal Brief dated May 31, 2007)	11
IX.	Evidence Appendices.	13
	Appendix C - Declaration by Dr. Allen S. Laughon dated December 9, 2005.	13
	Appendix D - Su et al. (2000) <i>Cancer Res.</i> 60:3137-3142.	29
X.	Related Proceedings Appendix.	35

I. Real Party in Interest

The real party in interest is Wisconsin Alumni Research Foundation.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of Claims

Claims 1-8 have been canceled.

Claims 9-12 are pending in this application.

Claims 9-12 have been rejected and are on appeal. Claim appendices including the text of the appealed claims (Appendix A), as well as proposed claim amendments (Appendix B), are attached.

IV. Status of Amendments

During the prosecution of this application, Appellant has filed multiple claim amendments, an affidavit by the inventor, and an RCE in an effort to respond to the Examiner's rejection of the claims under 35 U.S.C. 112, first paragraph. The basis of the instant invention is centered upon Appellant's appreciation that Smad proteins, DNA-binding Smad co-repressor proteins and CtBP proteins interact to mediate repression of genes that are negatively regulated by TGF- β signaling pathways. In this regard, the claimed assay employs a cell which co-expresses interacting proteins comprising a Smad protein, a DNA-binding Smad co-repressor protein and a CtBP protein and the use of a reporter with a promoter to identify agents that directly interact with the Smad protein or the DNA-binding Smad co-repressor protein to prevent protein-protein or protein-DNA interactions required for repression of transcription from genes induced by TGF- β , activin or bone morphogenetic protein signaling in cells.

In an effort to satisfy the written description requirement as to the method steps and reagents employed in the claimed assay, the

original claims were amended to include a reporter and a Smad box-containing promoter. Having maintained the written description rejection, Appellant attempted to further clarify the nature of the promoter by subsequently amending the claims to recite a TGF-beta-dependent promoter. This clarification was also rejected under 35 U.S.C. 112, first paragraph, and the claim was further amended to recite a promoter which is regulated by a TGF-beta, activin or bone morphogenetic protein signal (Appendix A). However, this amendment was also summarily rejected in the Final Office Action (mailing date November 30, 2006).

In response to this Final Rejection, Appellant made a further attempt to clarify the claimed assay by narrowing the scope of the claims to indicate that the promoter contained the response element TAGCCTGCCGTCGCGATTGACAACTTTGGCCGGCACGTTGGCGAGTGTGCCATGCATGCTGATGA (SEQ ID NO:5) (see response mailed February 16, 2007). In a first Advisory Action (mailing date March 20, 2007), the Examiner denied entry of this amendment because SEQ ID NO:5 had not been searched. Accordingly, Appellant responded to the first Advisory Action by proposing to limit the promoter to the specific wingless promoter, *i.e.*, a TGF-beta-regulated promoter (see Appendix B) (see response mailed March 29, 2007).

In the second Advisory Action (mailing date May 31, 2007), the Examiner again denied entry of the amendment and maintained the written description rejection because it was alleged that "a wingless promoter" had not been searched previously and was not of record in the specification. However, not only is a wingless promoter of record in the specification in page 7 (lines 19-23) and the paragraph spanning pages 9 and 10 and exemplified in Figure 6, the wingless promoter is also described as being a promoter which is regulated by the TGF-beta pathway (see the paragraph spanning pages 2 and 3). Therefore, being a species of the genus *TGF-beta-dependent promoter or promoter which is regulated by a TGF-beta*

signal, a wingless promoter was searched during the prosecution of this application.

The response to the Final Office Action (mailing date February 16, 2007) was not entered upon filing of this appeal.

V. Summary of the Claimed Subject Matter

Claim 9 defines a method for identifying compounds that directly interact with a Smad protein or a DNA-binding Smad co-repressor protein to prevent protein-protein or protein-DNA interactions required for repression of transcription from genes induced by TGF- β , activin or bone morphogenetic protein signaling in cells.

As disclosed at page 14, lines 13-21, an assay is contemplated for identifying proteins or small molecules that interact with Smad proteins to prevent interaction with CtBP with Smads or with DNA-binding co-repressors (e.g., Evi-1, TGIF, SIP1, or Schnurri), or the formation of a DNA-bound complex containing Smads, CtBP, and DNA-binding co-repressors, and thus prevent repression of genes that are negatively regulated by TGF β signaling pathways.

As in step (a) of claim 1, the paragraph bridging pages 14-15 specifies that the instant assay is a cell-based reporter assay, wherein compounds are screened based on their ability to disrupt CtBP-dependent repression by TGF- β *in vivo*. This passage further specifies the use of reporter proteins such as luciferase or beta-galactosidase. Moreover, page 8 (lines 10-16) teaches exemplary Smad proteins, page 14 (lines 14-17) teaches exemplary DNA binding protein Smad co-repressor proteins, and page 8 (lines 20-22) teaches exemplary CtBP proteins which interact with Smad and DNA binding protein Smad co-repressor proteins.

Step (a) of claim is exemplified at page 7 (lines 24-28) and Figure 6 which show the use of the LacZ (a reporter) under control of the Dpp-dependent (*i.e.*, TGF-beta-dependent) wingless promoter

in cells expressing Mad/Medea (*i.e.*, Smad proteins), Schnurri (*i.e.*, a DNA binding protein Smad co-repressor protein), and dCtBP.

As in step (b) of claim 1, page 15 (lines 5-6) discloses screening chemically diverse libraries of compounds in *in vivo* assays.

Page 15 (lines 2-5) and page 7 (lines 8-12) disclose steps (c) and (d) of claim 1, specifying that *in vivo* assays can use sensitive substrates in combination with the reporter to detect changes in TGF-beta-dependent reporter protein expression in response to specific compounds.

Moreover, the whole of the instant assay is exemplified at pages 9 and 10, which demonstrates co-expressing a Smad protein (*i.e.*, Mad and Medea), a DNA-binding Smad co-repressor protein (*i.e.*, Schnurri) and a CtBP protein (*i.e.*, dCtBP) in a cell (*i.e.*, *Drosophila* S2 cells) and detecting the level of transcription of a reporter (*i.e.*, LacZ) with a promoter (*i.e.*, a promoter containing a sequence from the wingless disc enhancer region (SEQ ID NO:5)) in the presence of a test compound (*i.e.*, Ci transcription factor). Furthermore, in a Declaration by Dr. Allen Laughon filed with the response mailed December 12, 2006 (attached hereto as Exhibit C), Appellant provided corroborative evidence that the guidance provided by the specification in combination with the well-known assay components successfully yielded the identification of the E1A protein as an inhibitor of CtBP-mediated repression of genes that are negatively regulated by TGF- β signaling pathways.

Exemplary embodiments of the DNA-binding Smad co-repressor protein as in claim 10 are disclosed at page 14 (lines 14-17). Likewise, claimed embodiments drawn *Drosophila* Mad or Medea (claim 11) are disclosed at page 8 (lines 10-16). Similarly, embodiments set forth in claim 12 are disclosed at page 8 (lines 20-22).

VI. Grounds of Rejection to be Reviewed on Appeal

Whether claims 9-12 should stand rejected under 35 U.S.C. §112, first paragraph, for failing to meet the written description requirement.

Whether claims 9-12 should stand rejected under 35 U.S.C. §112, second paragraph, for being indefinite.

VII. Arguments

A. The Rejection of Claims 9-12 Under 35 U.S.C. §112, First Paragraph Should Be Withdrawn

Appellant respectfully believes that this rejection is improper. On the one hand, the Examiner alleges that the specification does not provide adequate support for the claimed promoter, and on the other hand, the Examiner contends that the specification does not exemplify the claimed method. See page 3 of Final Rejection (mailing date November 30, 2006). Moreover, the Examiner concludes:

"And the specification does not exemplify an experimental design of the claimed assay. The steps listed in the claims are not of record in the specification. The claims do not meet the written description requirement because the specification is remiss of active method steps including cells containing interacting proteins and compounds necessitated for implementing the claimed method." See page 4 of the Final Rejection (mailing date November 30, 2006).

Thus, the Examiner's reasoning seems to be that the specification does not exemplify the method and therefore, written description is not met. However, lack of working examples is not an adequate basis for a written description rejection. See *Falkner v. Inglis*, 448 F.3d 1357, 1366, 79 USPQ2d 1001, 1007 (Fed. Cir. 2006) ("[E]xamples are not necessary to support the adequacy of a written description[;] ... the written description standard may be met ... even when actual reduction to practice of an invention is absent.").

MPEP 2163.02 quite clearly indicates that in order to comply with the written description requirement, the subject matter of the claim need not be described literally, i.e., using the same terms or *in haec verba*. What is required is that Appellant show possession of what is claimed. In viewing the whole of Appellant's disclosure, the written description requirement has been met. For example, Appellant discloses in the passage between page 14, line 11, and page 15, line 12, the principle of the claimed method, namely the identification of proteins or small molecules that interact with Smad proteins to prevent interaction of CtBP with Smads or DNA-binding Smad co-repressor proteins, or to prevent formation of a DNA-bound complex containing a Smad protein, CtBP protein and DNA-binding Smad co-repressor protein, thereby preventing repression of genes that are negatively regulated by TGF- β signaling pathways. As one embodiment, the assay is carried *in vivo* with a reporter protein having a TGF- β -dependent reporter (see the sentence bridging pages 14 and 15). In this regard, Appellant has exemplified the required elements of the claimed cell-based assay at pages 9 and 10. This disclosure teaches lacZ reporter constructs transfected into Drosophila cells and co-expressed with Schnurri (a DNA-binding Smad co-repressor protein) and Smads, Mad and Medea, which interact and repress transcription of the reporter. Furthermore, page 10 (lines 10-12) teaches that the repression of lacZ in transfected cells was enhanced by co-expression of dCtBP. It is clear from this disclosure that in order to achieve repression of the reporter, the Smad protein, CtBP protein and DNA-binding Smad co-repressor protein must be co-expressed by the cell being assayed. Thus, in contrast to the Examiner's suggestion, Appellant's disclosure clearly supports cells expressing interacting proteins and repression of the reporter.

Furthermore, Appellant respectfully disagrees with the Examiner's suggestion that the specification lacks support for the

claimed promoter. Appellant teaches at pages 1-3 that it is well-known in the art that TGF-beta, activin and bone morphogenetic protein signals regulate the expression of genes such as c-myc, cyclin, collagen proteases, and wingless. Indeed, Appellant exemplifies reporter constructs containing promoters (e.g., the wingless promoter), which are regulated by such signals in cell-based assays, wherein cells of the assays co-express interacting proteins comprising a Smad protein, a DNA-binding Smad co-repressor protein and a CtBP protein. See pages 9 and 10.

Moreover, to illustrate the established use of such promoters in screening assays, such as that presently claimed, Appellant provided in the response mailed April 23, 2005 the teachings of Su et al. ((2000) *Cancer Res.* 60:3137-3142; enclosed herewith as Exhibit D). Su et al. disclose the use of a reporter construct containing Smad-binding elements (p6SBE-luc) responsive to TGF- β signals in the presence and absence of inhibitors to measure processes that result in the nuclear localization of Smad4 (see, e.g., Figure 6). Using this screen, a novel histone deacetylase inhibitor, termed scriptaid, was identified which did not interfere with a further induction provided by stimulation of the cognate signal transduction pathway (i.e., TGF- β /Smad4). In light of the teachings of Su et al., Appellants respectfully believe that persons of skill in the art would readily know of and understand the claimed promoter which is regulated by a TGF- β , activin or bone morphogenetic protein signal. Thus, Appellant finds no basis for the Examiner's suggestion that persons skilled in the art would not recognize in Appellant's disclosure a description of the invention defined by the claims. Appellant believes that the essential features of the method of the invention (i.e., cells expressing Smad, CtBP, and DNA-binding Smad co-repressor proteins and a TGF- β -dependent reporter construct) are supported by the basic teachings of the disclosure and what is well-established in the art of

inhibitor screening with TGF- β -dependent reporter constructs. Thus, written description has been met.

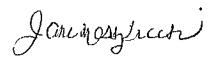
B. The Rejection of Claims 9-12 Under 35 U.S.C. §112, Second Paragraph Should Be Withdrawn

MPEP 2173.02 indicates that in reviewing a claim for compliance with 35 U.S.C. 112, second paragraph, the examiner must consider the claim as a whole to determine whether the claim apprises one of ordinary skill in the art of its scope and, therefore, serves the notice function required by 35 U.S.C. 112, second paragraph.

In this regard, it is respectfully submitted that for the reasons indicated above, the skilled artisan would clearly understand what is meant by a TGF- β , activin or bone morphogenetic protein signal. Moreover, Appellant respectfully points out that page 4 (lines 19-31) discloses that compounds identified by the instant assay interfere with transcriptional repression that otherwise would occur in response to signaling by a TGF β , activin or bone morphogenetic protein. As such, it is clear from this disclosure that a TGF- β , activin or bone morphogenetic protein signal includes a TGF β , activin or bone morphogenetic protein. Therefore, it is respectfully submitted that the instant claims are not indefinite.

Accordingly, reversal of the Examiner's rejections of claims 9-12 under 35 U.S.C. §112 first and second paragraphs is therefore respectfully requested.

Respectfully submitted,



Jane Massey Licata
Registration No. 32,257

Date: January 15, 2008

Licata & Tyrrell P.C.
66 East Main St.
Marlton, NJ 08053

(856) 810-1515

IX. Evidence Appendices - Appendix C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No. : WARF-0002

Inventors: Laughon, Allen S.

Serial No. : 09/810,385

Filing Date: March 16, 2001

Examiner: Harris, Alana M.

Customer No. : 26259

Group Art Unit: 1643

Confirmation No. : 8778

Title: Compositions and Methods for Negative Regulation of TGF- β Pathways

Express Mail Label No. EV794825581US
Date of Deposit December 12, 2005

I hereby certify that this paper is being deposited with the United States Postal Service Express Mail Post Office to Addressee service under 37 CFR 1.10 on the date indicated above and is addressed to the Mail Stop AF Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

By Laughon, Allen S.
Typed Name: Jane Massey Licata, Reg. No. 32,257

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RULE 132 DECLARATION

1. I, Dr. Allen S. Laughon, Ph.D. am the inventor in U.S. Patent Application Serial No. 09/810,385 filed March 16, 2001 and am most familiar with the subject matter of this application and the research effort which lead to the discovery of the instant invention.

2. As described in the '385 application at pages 14 and 15, a cell-based reporter assay has been used to identify compounds that interfere with transcriptional repression of genes induced by a TGF- β , activin or bone morphogenetic protein signal in cells. The components

VIII. Claims Appendices - Appendix A - Pending Claims

Claims 1-8 (canceled).

Claim 9 (previously presented): A method for identifying compounds that directly interact with a Smad protein or a DNA-binding Smad co-repressor protein to prevent protein-protein or protein-DNA interactions required for repression of transcription from genes induced by TGF- β , activin or bone morphogenetic protein signaling in cells comprising:

(a) detecting in a cell a first level of transcription of a reporter with a promoter which is regulated by a TGF- β , activin or bone morphogenetic protein signal, wherein said cell co-expresses interacting proteins comprising a Smad protein, a DNA-binding Smad co-repressor protein and a CtBP protein;

(b) contacting said cell with a test compound;

(c) detecting a second level of transcription of the reporter in the cell after addition of the test compound; and

(d) comparing the first level with the second level, wherein a decrease in the level of repression of transcription of the reporter in said cell after addition of the test compound is indicative of the ability of the test compound to interfere with transcriptional repression of genes induced by a TGF- β , activin or bone morphogenetic protein signal in cells.

Claim 10 (previously presented): The method of claim 9 wherein transcription levels of the reporter both before and after addition of the test compound are detected in cells expressing a Smad protein, a CtBP protein, and a DNA-binding Smad co-repressor protein selected from the group consisting of Evi-1, TGIF, SIP1, and Schnurri.

Claim 11 (previously presented): The method of claim 9 wherein the Smad protein is *Drosophila* Mad or Medea.

Claim 12 (previously presented): The method of claim 9 wherein the CtBP protein is dCtBP or CtBP2.

Appendix B - Proposed Claim Amendments (offered in Response to Advisory Action but not entered per Advisory Action Before Filing of an Appeal Brief dated May 31, 2007)

Claims 1-8 (canceled).

Claim 9 (not entered): A method for identifying compounds that directly interact with a Smad protein or a DNA-binding Smad co-repressor protein to prevent protein-protein or protein-DNA interactions required for repression of transcription from genes induced by TGF- β , activin or bone morphogenetic protein signaling in cells comprising:

(c) detecting in a cell a first level of transcription of a reporter with a wingless promoter ~~which is regulated by a TGF β , activin or bone morphogenetic protein signal~~, wherein said cell co-expresses interacting proteins comprising a Smad protein, a DNA-binding Smad co-repressor protein and a CtBP protein;

(d) contacting said cell with a test compound;

(c) detecting a second level of transcription of the reporter in the cell after addition of the test compound; and

(d) comparing the first level with the second level, wherein a decrease in the level of repression of transcription of the reporter in said cell after addition of the test compound is indicative of the ability of the test compound to interfere with transcriptional repression of genes induced by a TGF- β , activin or bone morphogenetic protein signal in cells.

Claim 10 (previously presented): The method of claim 9 wherein transcription levels of the reporter both before and after addition of the test compound are detected in cells expressing a Smad protein, a CtBP protein, and a DNA-binding Smad co-repressor protein selected from the group consisting of Evi-1, TGIF, SIP1, and Schnurri.

Claim 11 (previously presented): The method of claim 9 wherein the Smad protein is *Drosophila* Mad or Medea.

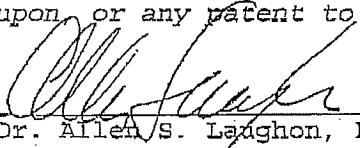
Claim 12 (previously presented): The method of claim 9 wherein the CtBP protein is dCtBP or CtBP2.

Attorney Docket No.: WARF-0002
Inventors: Laughon, Allen S.
Serial No.: 09/810,385
Filing Date: March 16, 2001
Page 2

used in the assay were known at the time of filing of the '385 application and are those described in the '385 application and depicted in Figure 6, namely Mad and Medea as Smad proteins, Shn as the DNA-binding Smad co-repressor protein, dCtBP, and lacZ as the reporter, the expression of which is controlled by the *brk* promoter, a direct target of Mad/Medea and Schnurri (see abstract of Muller et al. (2003) *Cell* 113:221– 233, Exhibit A).

The results of this assay (see Exhibit B) show that a mutant version of the adenovirus E1A protein (designated in Exhibit B as pPACELA), which is a documented inhibitor of CtBP, blocks repression of the *brk*-lacZ reporter construct in transiently transfected Drosophila S2 cells. In this assay, repression of the *brk*-lacZ reporter is caused by cotransfection of a plasmid that expresses TkvQD, an activated form of the type I Dpp receptor. Cotransfection with a plasmid expressing E1A blocks this repression by inhibiting CtBP. Accordingly, having used the well-known assay components and guidance provided in the specification for carrying out the method of the '385 application, we have successfully identified a compound that interferes with transcriptional repression of genes induced by a TGF-13, activin or bone morphogenetic protein signal in cells.

I hereby declare that all statements herein of our own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements were made with the knowledge that willful statements and the like so made are punishable by fine or by imprisonment, or both under §1001 of Title 18 or the United States Code, and that such willful statements may jeopardize the validity of the application, any patent issuing thereupon or any patent to which this verified statement is directed.


Dr. Allen S. Laughon, Ph.D.

Date: Dec. 9, 2005

Conversion of an Extracellular Dpp/BMP Morphogen Gradient into an Inverse Transcriptional Gradient

Bruno Müller,^{1,3} Britta Hartmann,^{2,3}
George Pyrowolakis,^{2,3} Markus Affolter,^{2,*}
and Konrad Basler^{1,*}

¹Institut für Molekularbiologie
Universität Zürich

Winterthurerstrasse 190
CH-8057 Zürich

²Abteilung Zellbiologie
Biozentrum der Universität Basel
Klingelbergstrasse 70
CH-4056 Basel
Switzerland

the anteroposterior compartment boundary and exerts a direct and long-range organizing influence on both the anterior and posterior halves (reviewed by Strigini and Cohen, 1999; Podos and Ferguson, 1999). In addition to controlling growth, Dpp induces the expression of different target genes above distinct threshold concentrations. These targets include *vestigial* (*vg*), *optomotor-blind* (*omb*), and *spalt* (*sal*), are expressed in progressively narrower domains, define the primordium of the wing blade, and control important aspects of pattern, differentiation, and survival (Kim et al., 1996; Grimm and Pflugfelder, 1996; Sturtevant et al., 1997; reviewed by Podos and Ferguson, 1999).

An understanding of how morphogen gradients operate requires answers to two different questions. How do concentration gradients arise, and how do cells interpret different morphogen concentrations? While recent efforts in the field focused on the problem of how Dpp protein spreads through tissue (Ramírez-Weber and Kornberg, 1999; Entchev et al., 2000; Teleman and Cohen, 2000), we are here concerned with the question of how a Dpp gradient is converted into transcriptional outputs.

Like all members of the TGF β superfamily, Dpp assembles at the cell surface a receptor serine/threonine kinase complex comprising subunits known as the type I and type II receptors, encoded by the genes *thick veins* (*tkv*) and *punt*, respectively (reviewed by Massagué, 1998; Podos and Ferguson, 1999; Tabata, 2001). The binding of Dpp to its receptors triggers the phosphorylation of Tkv by Punt and in turn enables Tkv to recognize and phosphorylate the Smad protein Mad (Raftery and Sutherland, 1999; Tanimoto et al., 2000). Phosphorylation releases Mad from cytoplasmic retention, allowing its association with the related factor Medea (Med) (Hudson et al., 1998; Inoue et al., 1998; Wisotzkey et al., 1998) and subsequent translocation into the nucleus, where the two proteins are involved in the transcriptional regulation of target genes (reviewed by Massagué and Wotton, 2000; Affolter et al., 2001).

Mad and Med possess DNA binding activities that have been implicated in the recognition of a regulatory element in the *vg* gene (Kim et al., 1997). Hence, *Drosophila* Smad proteins have been proposed, in analogy to their vertebrate counterparts, to directly activate the Dpp targets *vg*, *omb*, and *sal*. An alternative mechanism has recently emerged with the unexpected discovery of Brinker (Brk), a transcription factor that is required to counteract responses to Dpp (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999). Loss of Brk function causes overproliferation and ligand-independent, ectopic expression of the Dpp targets *vg*, *omb*, and *sal*. *brk* expression itself is negatively regulated by Dpp, such that peripheral cells in the wing disc express high and central cells undetectable levels of Brk. These findings raise the possibility that it is primarily the repressive function of Brk that controls growth and Dpp target gene expression and that direct transcriptional activation by Mad may only play a subordinate role.

Summary

Morphogen gradients control body pattern by differentially regulating cellular behavior. Here, we analyze the molecular events underlying the primary response to the Dpp/BMP morphogen in *Drosophila*. Throughout development, Dpp transduction causes the graded transcriptional downregulation of the *brinker* (*brk*) gene. We first provide significance for the *brk* expression gradient by showing that different Brk levels repress distinct combinations of wing genes expressed at different distances from Dpp-secreting cells. We then dissect the *brk* regulatory region and identify two separable elements with opposite properties, a constitutive enhancer and a Dpp morphogen-regulated silencer. Furthermore, we present genetic and biochemical evidence that the *brk* silencer serves as a direct target for a protein complex consisting of the Smad homologs Mad/Medea and the zinc finger protein Schnurri. Together, our results provide the molecular framework for a mechanism by which the extracellular Dpp/BMP morphogen establishes a finely tuned, graded read-out of transcriptional repression.

Introduction

It was proposed more than a century ago that the organization of cell and body patterns might be controlled by concentration gradients of “form-producing” substances or morphogens (Morgan, 1897; Turing, 1952; Wolpert, 1989). Only recently has it been possible to demonstrate that secreted proteins of the Wnt, Hedgehog, and transforming growth factor- β (TGF β) families specify positional information by this mechanism (reviewed by Gurdon and Bourillot, 2001).

Particularly compelling evidence for the existence of an extracellular morphogen gradient comes from studies on the developing wing imaginal disc of *Drosophila*, where a localized source of the BMP2/4 homolog Decapentaplegic (Dpp) is expressed in a stripe of cells along

*Correspondence: konrad.basler@molbio.unizh.ch (K.B.), markus.affolter@unibas.ch (M.A.)

³These authors contributed equally to this work.

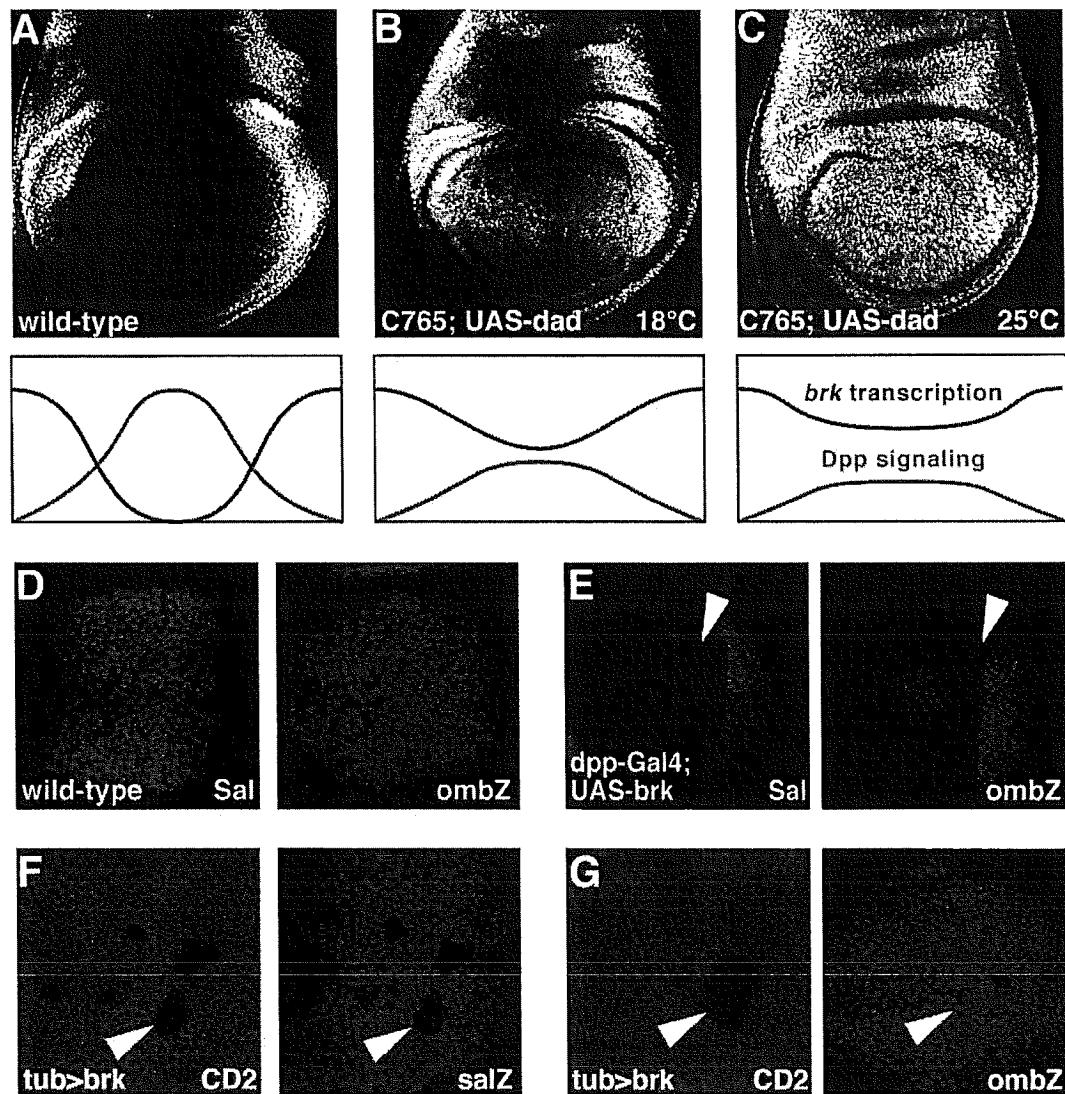


Figure 1. The Dpp Signaling Gradient Determines the Profile of *brk* Expression, Which in Turn Defines the Activity States of Dpp Target Genes

(A-C) Confocal sections of wing discs are shown (dorsal up, anterior to the left). *brk* expression is visualized by means of the *brk-lacZ* reporter X47 in wild-type (A) and when Dpp signaling is downregulated by expressing the inhibitory Smad *Daughters against dpp* (*Dad*, [B and C]). The low-level ubiquitous C765-*Gal4* line was used to induce a *UAS-dad* transgene, which results in a shallower, and hence better detectable, Dpp gradient. Below each panel our interpretation is shown in the form of diagrams, which indicate the inverse relation between Dpp signaling levels (red) and *brk* expression levels (blue).

(D-G) Different Brk levels define distinct combinations of target gene expression. (D) Wild-type expression patterns of the Dpp target genes *sal* (in green) and *omb* (in red) are shown in wing primordia. (E) High levels of ectopic Brk expression were obtained with *dpp-Gal4 UAS-brk*. These levels of Brk repress the expression of both *sal* (E, left) and *omb* (E, right). The domain of *dpp-Gal4* activity is broader than the domain of endogenous *dpp* expression, hence the widespread effect in the anterior compartment. *dpp-Gal4*, rather than *actin5c>Gal4*-expressing clones, was used in this experiment to drive *UAS-brk* expression, because clones ectopically expressing substantial levels of *brk* rapidly undergo apoptosis in the wing pouch epithelium. (F and G) Low levels of ectopic Brk were obtained with a *tubulinα1>CD2>brk* construct. Clones expressing *brk* under the *tubulinα1* promoter (*tub>brk*) are marked by the absence of CD2 staining (in green). In such clones, *sal* expression is repressed (F), but *omb* expression is unaffected (G).

Here, we provide strong support for this view by studying the role and establishment of the Brk gradient. We find that the output of Dpp signaling and the action of the zinc finger protein Schnurri (Shn), both of which have been implicated by genetic means in the regulation of *brk* (Marty et al., 2000; Torres-Vazquez et al., 2000), converge on defined silencer elements of *brk*. The re-

sulting inverse expression gradient of nuclear Brk protein has the capacity to differentially regulate *omb* and *sal*. Our results provide the molecular framework for a mechanism in which the extracellular Dpp gradient is converted into primary nuclear outputs via the generation of an inverse transcriptional gradient of *brk* by means of Shn-dependent silencer elements.

Results

Dpp Signaling Levels Control the Profile of the Brk Expression Gradient

High levels of Dpp signaling prevent the expression of the *brk* gene (Campbell and Tomlinson, 1999; Minami et al., 1999). In contrast, *brk* is readily transcribed in cells situated far away from a Dpp source or in cells with an experimental block in the Dpp transduction pathway (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999). In leg and wing imaginal discs, lateral cells, expressing maximal levels of *brk*, and central cells, in which *brk* expression cannot be detected, are separated by a seemingly narrow stripe of cells with graded *brk* expression. To explore whether position and spatial extent of this population are sensitive to Dpp signaling levels, we altered the presumptive Dpp signaling gradient by ubiquitously expressing the inhibitory Smad6 homolog Dad (Tsuneizumi et al., 1997). Low Dad levels cause a significant expansion of the *brk*-expressing domains toward the center of the disc (Figure 1B, C765-Gal4 UAS-dad at 18°C) with an extended, shallow gradient of *brk* levels. Higher levels of Dad (Figure 1C, same genotype at 25°C) produce an even more pronounced effect with cells along the entire anteroposterior (AP) axis expressing *brk*. We interpret these observations as indication that different levels of Dpp signaling determine, with an inverse relationship, different levels of *brk* expression. These experiments taken together with the genetic requirement of *brk* for regulating target genes (data not shown) suggest that the functional Brk gradient extends beyond the domain in which graded *brk* expression can be detected with reporter genes in wild-type.

Brk Expression Levels Control the Activity States of Dpp Target Genes

The Dpp target genes *vg*, *omb*, and *sal* are expressed in nested domains with progressively narrower widths of activity along the AP axis. The expression of all three of these genes is subject to repression by Brk in lateral regions of the wing disc (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999), raising the possibility that different levels of *brk* alone are able to specify distinct combinations of activity states of these genes. To address this possibility, we asked whether low levels of ectopic Brk expression can repress *sal*, but not *omb*, transcription, whereas high levels of Brk levels would repress both genes. High levels of ectopic Brk expression in the center of the disc were obtained by using a *UAS-brk* transgene in conjunction with a *dpp-Gal4* driver. Low levels of Brk were expressed by the weak constitutive promoter from the *tubulin α 1* gene in marked clones of cells. As shown in Figure 1E, the *dpp-Gal4 UAS-brk* transgenes cause repression of both *sal* and *omb* transcription. In contrast, the lower levels of *brk* produced by *tubulin α 1>brk* repress only *sal*, while *omb* transcription is not affected. Hence, different levels of Brk expression can elicit distinct outputs.

Together, the experiments described so far imply that the transcriptional control of *brk* is a key event in the interpretation of the Dpp morphogen gradient. In order to understand how this morphogen gradient becomes

translated into different cell fates, we focused on the question of how Dpp generates an inverse transcriptional gradient of *brk* expression.

Dissection of the *brk* Regulatory Regions into Separable Enhancer and Repression Activities

Our first efforts were directed toward isolating the regulatory elements of the *brk* gene that ensure proper expression levels along the AP axis in response to Dpp signaling. We scanned the 20 kb region between the *brk* transcription unit and its upstream neighboring locus for such elements (Figure 2A). Restriction fragments from genomic *lambda* phages were cloned into a *lacZ* reporter P element and assayed for regulatory activity in vivo. This led to the identification of fragment B14, which faithfully recapitulates all aspects of late embryonic and larval *brk* expression (Figure 2A).

Interestingly, we found that distal truncations of B14 caused a progressive widening of the lateral expression domains toward the center of wing imaginal discs, while the levels of expression remained constant (Figure 2A). This observation suggested to us that the *brk* enhancer consists of two separable entities, a ubiquitously active, constitutive enhancer element located in the proximal half and a regulated repression activity encoded by the distal half.

Both activities were narrowed down by an extensive series of reporter constructs, a small subset of which is shown in Figures 2B–2D (for details, see legend to Figure 2). Three short fragments (called A, B, and C) were identified that possess repression activities when coupled to the constitutive enhancer represented by construct B38 (Figures 2B and 2C). The most potent of these short elements, fragment C, was further dissected into a 53 bp element (Figure 2D), referred to as S (S for silencer, see below). Its repression function is encoded in a nonredundant manner, as point mutations abolish its activity (Figure 2D and Experimental Procedures).

The dissection of 20 kb of potential regulatory sequences into two discrete minimal elements with opposite activities, which together reconstitute the hallmarks of *brk* expression, establishes the basis for our molecular studies. As described below, fragment C and its shorter derivative S serve as a paradigm to study the regulation of *brk* repression.

A Signaling-Regulated Silencer: The *brk* Repression Element Can Operate Independently of the *brk* Enhancer, but Its Activity Depends Strictly on Dpp Input

The activities of the *brk* regulatory elements were analyzed in diverse imaginal and embryonic tissues (Figure 3). Invariably, repression activity was maximal in vicinity of well-characterized sources of Dpp, suggesting that this activity is dependent on Dpp signaling. To confirm this apparent requirement for Dpp input, the repression activity was monitored in wing disc cells lacking the Dpp type I receptor Thick-veins (Tkv). *tkv* mutant cells autonomously lost repression activity (data not shown, but see below), indicating that this repression is strictly regulated by Dpp signaling.

Reporter constructs exhibiting spatially decreased domains of repression can therefore be regarded as less

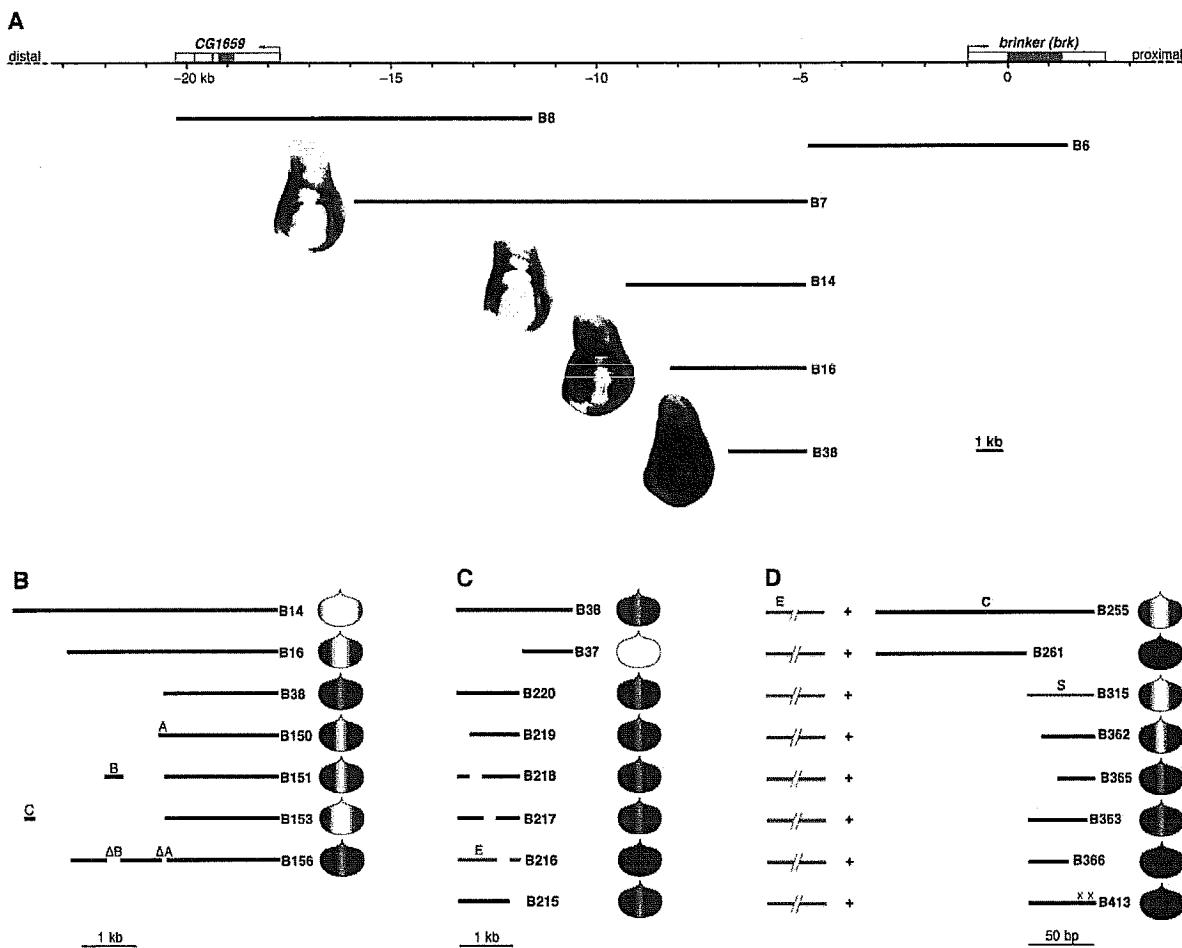


Figure 2. Dissection of the *brk* Regulatory Region into Separable Activating and Repressing Activities

(A) A map of the upstream region of the *brk* locus is shown on top. Restriction fragments B6, B7, and B8 were tested for their ability to drive reporter gene expression in transgenic animals. Fragment B8 did not cause any detectable expression, and no transgenic animals were obtained from fragment B6, likely due to toxicity. Fragment B7 faithfully recapitulated all aspects of *brk* expression and was further reduced in size, leading to the 5 kb fragment B14 that still drives *brk*-like expression. Distal truncations of fragment B14 resulted in a progressive widening of the lateral expression domains in imaginal discs (see B14, B16, and B38), suggesting that B14 contains repression elements in its distal part and a constitutively active enhancer in its proximal part (represented by fragment B38). In all panels, only a small subset of the constructs tested are shown.

(B) Three fragments A, B and C were identified in the distal part of B14 to cause repression in central regions of the wing disc (in combination with the constitutively active enhancer B38), as shown in constructs B150, B151, and B153. Among the three fragments, C showed strongest activity. If sequences A and B are removed from B16 (i.e., construct B156) repression activity is almost completely lost and expression is like that of B38.

(C) Fragment B38 still shows slightly reduced expression in the center of the disc where Dpp signaling is highest. In an attempt to obtain an enhancer fragment that is uniformly expressed, B38 was further dissected. This lead to the identification of B216 (shown in green) that is evenly and ubiquitously expressed in the wing pouch and therefore provides a sensitive tool to test other fragments for their ability to mediate regulated repression. We call B216 "E" for *enhancer*.

(D) Fragment C (see Figure 2B) was chosen for further analysis. The repressive activity of C and of its derived subfragments was assayed in combination with E (Figure 2C), and was localized to a 53 bp subfragment, referred to as "S" (for *silencer*, shown in red). The activity of S was strongly reduced and became unstable by further terminal deletions (B362, B365, B363, and B366). In addition, systematic point mutations throughout S identified base pairs that are essential for repression activity (exemplified by B413), leading us to conclude that S represents a minimal fragment. For nucleotide sequences of S and B413, see Experimental Procedures.

sensitive toward Dpp input. In all tissues examined, the decrease in sensitivity of such reporters is similar to that observed in wing discs (Figure 3), indicating that the *brk* repression element operates throughout embryonic and imaginal stages to perceive the activity state of the Dpp signal transduction pathway.

So far, the *brk* repression element has only been assayed in the context of the constitutive *brk* enhancer, which is part of the same regulatory region in the *Drosophila*

sophila genome. We sought to test whether this negative regulatory element can impose Dpp-dependent repression on heterologous enhancers. Below, we use three diverse enhancers in three different systems to provide evidence that this is indeed the case.

First, we used a previously characterized regulatory element of the *dpp* locus, which directs uniform expression within the pouch region of wing imaginal discs (Müller and Basler, 2000). When the *brk* repression ele-

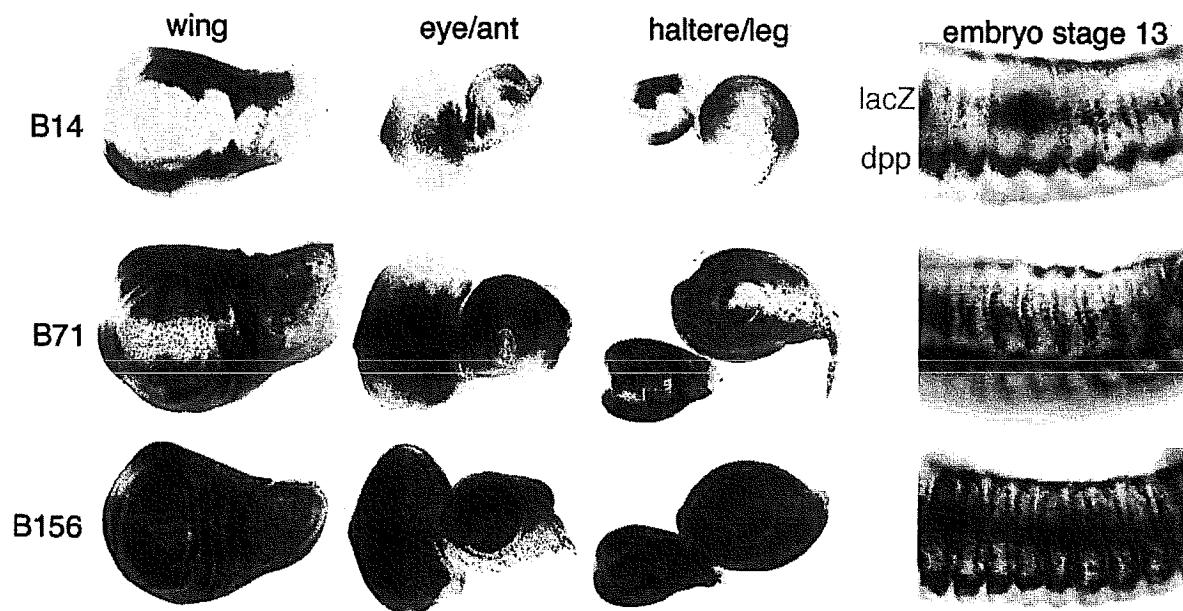


Figure 3. Throughout Embryonic and Larval Tissues, the Activity State of the Dpp Signaling System Is Integrated by the *brk* Regulatory Region
Expression of *brk* reporter constructs with different sensitivities to Dpp signaling are shown: wild-type sensitivity (B14, top row), decreased sensitivity (B71, middle row), and almost absent sensitivity (B156, lowest row). In all larval tissues analyzed (i.e., eye, antennal (ant), haltere, and leg imaginal discs), as well as in mid- and late-embryonic tissues, the sensitivity of the reporter constructs to Dpp is similar to that observed in wing discs, judged by the gap between the expression domains of *dpp* (data not shown for discs) and the reporters. In the embryo (the rightmost panels) *dpp* mRNA expression is shown in blue, and the antibody staining in brown detects the *lacZ* expression of the reporter constructs. Wing discs are oriented with their anterior side up and dorsal to the right.

ment is linked to this enhancer, transcriptional activity is confined to the lateral edges of the wing pouch (Figure 4A). Second, we assayed the embryonic *even-skipped-stripe-2* enhancer (Small et al., 1992) in isolation of, and combination with, the *brk* repression element. This enhancer is normally active in a circumferential band of cells in the early blastoderm stage. However, when linked to *brk* repression elements, the *even-skipped-stripe-2* enhancer is repressed in dorsal regions of the embryo (Figure 4B), where cells are exposed to high levels of Dpp (Ferguson and Anderson, 1992). Finally, we assayed the activity of the *brk* repression element in the context of a Notch-responsive enhancer in *Drosophila* tissue-culture cells. This synthetic enhancer shows a 30- to 40-fold stimulation of reporter gene expression upon transfection of S2 cells with plasmids driving the expression of Suppressor of Hairless (Su[H]) and a constitutively active form of Notch (Kirkpatrick et al., 2001). Simultaneous cotransfection of a plasmid encoding the activated form of the Dpp receptor Tkv (Tkv^{QD}, see Nellen et al., 1996) blocks this activation, in a manner strictly dependent on the presence of the *brk* repression fragment (Figure 4C). Thus, S2 cells are capable of transducing Tkv input and converting it into transcriptional regulation. The repression mediated by the *brk* element in this system occurs in the context of a heterologous enhancer located on transfected plasmid DNA.

Together with our finding that the *brk* repression element can mediate Dpp-dependent repression independently of its orientation or position (data not shown), the above-described results allow us to call this element a "signaling-regulated silencer."

The Net Balance of Silencer and Enhancer Activities Determines the *brk* Expression Levels

The results presented so far indicate that the levels of *brk* expression determine the fate of wing cells along the AP axis and that these levels are defined by three parameters: (1) the degree of activation of the Dpp transduction pathway, (2) the "strength" of the constitutive *brk* enhancer, and (3) the repressive activity of the *brk* silencer at any given degree of Dpp signaling. This model raises the prediction that altering any of the three parameters, while leaving the other two fixed, should have a direct impact on the spatial profile of *brk* expression. In the first result section, we have tested the effects of altering the Dpp signaling levels. We next set out to alter the activity of the *brk* silencer (S). As shown in Figure 5, an increase of its copy number results in a progressive lateral shift of those cells that express high detectable levels of reporter gene activity in the wing disc. Conversely, the duplication of the constitutive *brk* enhancer (E) has the opposite effect and leads to an expansion of reporter gene expression toward the disc center at a given number of *brk* silencer elements. Hence, it is the net balance of the two opposing regulatory forces that determines the level of *brk* expression at any given level of Dpp signaling.

brk Silencer Activity Depends on Mad, Med, and Schnurri Function

Over the past few years, a fairly detailed picture has emerged of how target genes are activated in response to ligands of the TGF β , BMP, and Activin families in a stage- and tissue-specific manner (reviewed by Mas-

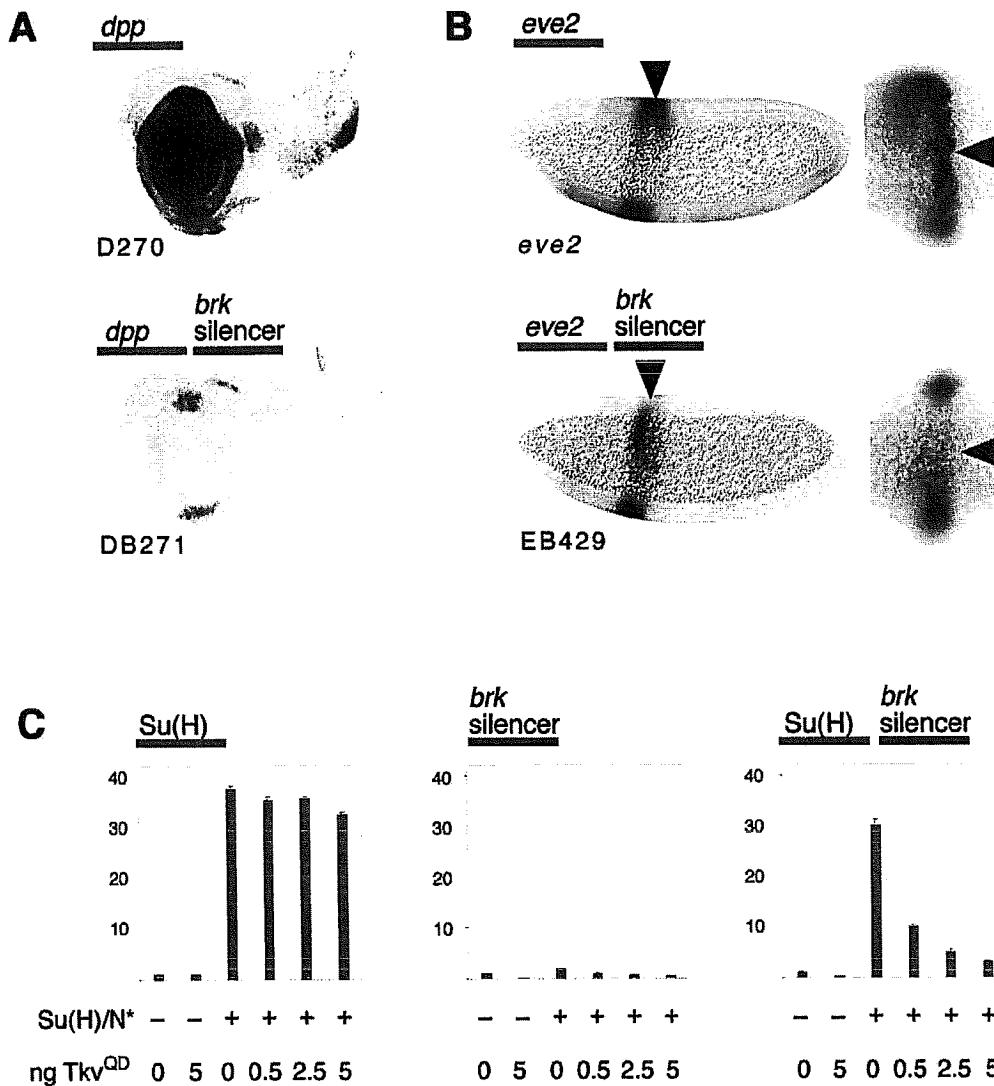


Figure 4. The *brk* Silencer Imposes Repression on Heterologous Enhancers Active in Discs, in the Early Embryo, and in S2 Cells

(A) Construct D270 is derived from a minimal *dpp* enhancer that lacks repressive inputs of both Ci and Engrailed (see Figure 2 of Müller and Basler, 2000) and drives reporter expression in the entire wing pouch. The addition of *brk* silencer fragments in construct DB271 leads to a repression in central domains where Dpp signaling occurs.

(B) A blastoderm-stage embryo in which reporter gene expression is driven by the even-skipped-stripe-2 enhancer (*eve2*, Small et al., 1992) is shown on top, either in a lateral view (to the left) or dorsal view (to the right, higher magnification). The *eve2* enhancer is fully active on the dorsal side of the embryo (arrowheads). The addition of *brk* silencer elements (construct EB429) causes a repression in dorsal domains where Dpp signaling is highest (arrowheads). The anterior sides of embryos are oriented to the left.

(C) β -galactosidase reporter assays in *Drosophila* S2 cells. Reporter plasmids contain the *lacZ* gene under the control of a Suppressor of Hairless response element (*Su(H)*, in green, left), a fragment of the *brk* control region containing the subfragment S (*brk* silencer, in red, middle), or the combination of the two elements (right). These reporters were cotransfected with a combination of plasmids encoding *Su(H)* and an activated form of Notch (*N**). Increasing amounts of a plasmid expressing *Tkv*^{QD} lead to a stepwise repression of reporter activity (right). *Tkv*^{QD}-mediated repression is strictly dependent on the presence of the *brk* silencer since it is not observed with the reporter containing only the *Su(H)* response element (left). β -galactosidase values were normalized by cotransfected 5 ng of a plasmid expressing luciferase as an internal standard. Results shown represent the average β -galactosidase activities from transfections done in triplicates (\pm standard deviation) and are expressed as the X-fold activation over the basal activity of each reporter plasmid alone.

sagué and Wotton, 2000; Attisano and Wrana, 2002). To explore how input by the BMP homolog Dpp causes repression rather than activation of *brk* transcription, and how it can do so in virtually all cells of an organism, we set out to analyze this process by genetic and biochemical means. We first assayed the requirements for the known Dpp signal transduction components in the

above-described context in which the *brk* silencer represses transcription driven by the heterologous wing blade enhancer from the *dpp* locus. As shown in Figures 6A–6C, wing cells require the activities of the *tkv*, *Mad*, and *Med* genes to repress reporter gene expression. In addition, the *brk* silencer also depends on *Shn* function, as *shn* mutant cells ectopically express high levels of

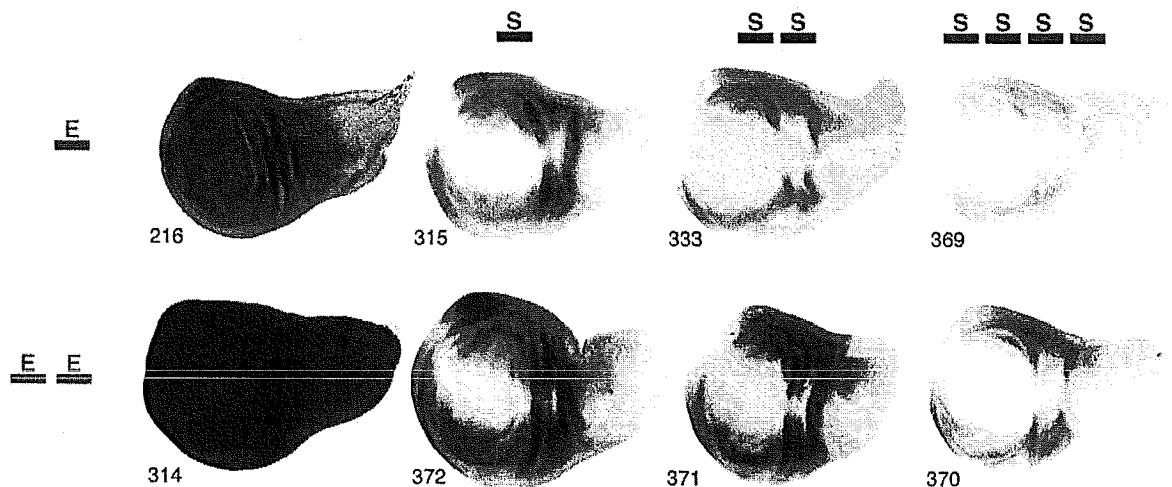


Figure 5. The Net Balance of *brk* Enhancer and Silencer Activities Determines the Transcriptional Output Levels in a Given Cell

None, one, two, or four copies of *brk* silencer elements (S, shown in red) were combined with one (top row) or two (bottom row) *brk* enhancer elements (E, shown in green). An increase in the copy number of S results in a progressive lateral shift of those cells that express high detectable levels of reporter gene activity (both rows, from left to right). Conversely, the duplication of enhancer element E has the opposite effect and leads to an expansion of reporter gene expression at a given number of *brk* silencer elements (compare top row with bottom row). Discs are oriented with their anterior side up.

the reporter gene (Figure 6D). The same requirements were observed in cultured cells, where *Tkv*^{0D} activity was no longer able to abolish Notch-induced activation of reporter gene expression when either endogenous *Mad* or endogenous *shn* functions were knocked-down by RNAi (Figure 6F). Addition of either double-stranded RNA had no effect, however, on the Notch-stimulated induction in the absence of Dpp signaling.

The C-Terminal 640 Amino Acids of Shn Are Necessary and Sufficient for Dpp-Dependent *brk* Repression In Vivo and In Vitro

Our observations that repression by the minimal *brk* silencer shows the same requirements in S2 cells as in vivo (i.e., an activated Dpp receptor, *Mad*, *Med*, and *Shn*) prompted us to analyze this process biochemically with epitope-tagged proteins. However, all our attempts to detect significant amounts of full-length Shn protein in extracts from embryos or from transfected S2 cells failed. The Shn protein is very large (2529 amino acids, Arora et al., 1995; Grieder et al., 1995) and proved to be refractory to biochemical manipulation in our hands. To overcome this limitation, we searched for shorter derivatives of Shn that retained the ability to mediate Dpp-dependent repression of *brk*. In a series of Shn proteins with terminal truncations and/or internal deletions, we identified one short form, referred to as ShnCT, which retained the key properties of full-length Shn. Like transgene-derived full-length Shn, ShnCT is able to repress transcription of the endogenous *brk* gene (data not shown), as well as that of the B14 reporter gene (Figure 7A), in *shn* null mutant embryos in a Dpp-dependent manner. ShnCT comprises the C-terminal 640 amino acids and thus three of the eight Shn zinc finger motifs. In contrast, ShnNT, which comprises all but the 640 residues of ShnCT, or ShnZF6-8, which

only lacks the three C-terminal zinc fingers, has no detectable rescuing activity (Figure 7A), indicating that these structural motifs play a crucial role in repression via *brk* silencer elements.

To confirm that the same C-terminal Shn sequences are able to mediate *brk* repression in our S2 cell assay, we expressed ShnCT in cells treated with double-stranded RNA against the central portion of endogenous *shn* mRNA and, hence, substituted endogenous Shn protein with ShnCT. Dpp-dependent repression was fully recovered under these conditions (Figure 7B). Furthermore, and as observed in vivo, the three clustered zinc fingers in ShnCT are critical for this rescue of repression, validating our Shn reagent as well as our cell-based transcription assay.

The *brk* Silencer Element Assembles a Shn/Mad/Med Complex

Since all three proteins that are required for Dpp-dependent repression by genetic criteria contain putative DNA binding domains (i.e., *Mad*, *Med*, and *Shn*), we set out to test their ability to molecularly interact with the *brk* silencer element in extracts of *Tkv*^{0D}-expressing S2 cells. Electrophoretic mobility shift assays indicated that neither ShnCT nor *Med* was able to form a stable protein/DNA complex (Figure 7C), although both proteins were readily expressed (data not shown). Transfection with a *Mad*-encoding plasmid resulted in the formation of a detectable protein/DNA complex (Figure 7C, lane 3), but a more prominent complex of similar mobility was obtained upon expression of *Mad* in combination with *Med* (Figure 7C, lane 7). Coexpression of ShnCT with *Mad* and *Med* led to the formation of a complex of even slower mobility (lane 8), suggesting that ShnCT is recruited to the *brk* silencer element with the help of *Mad* and *Med*. In contrast, the complex that formed in the presence

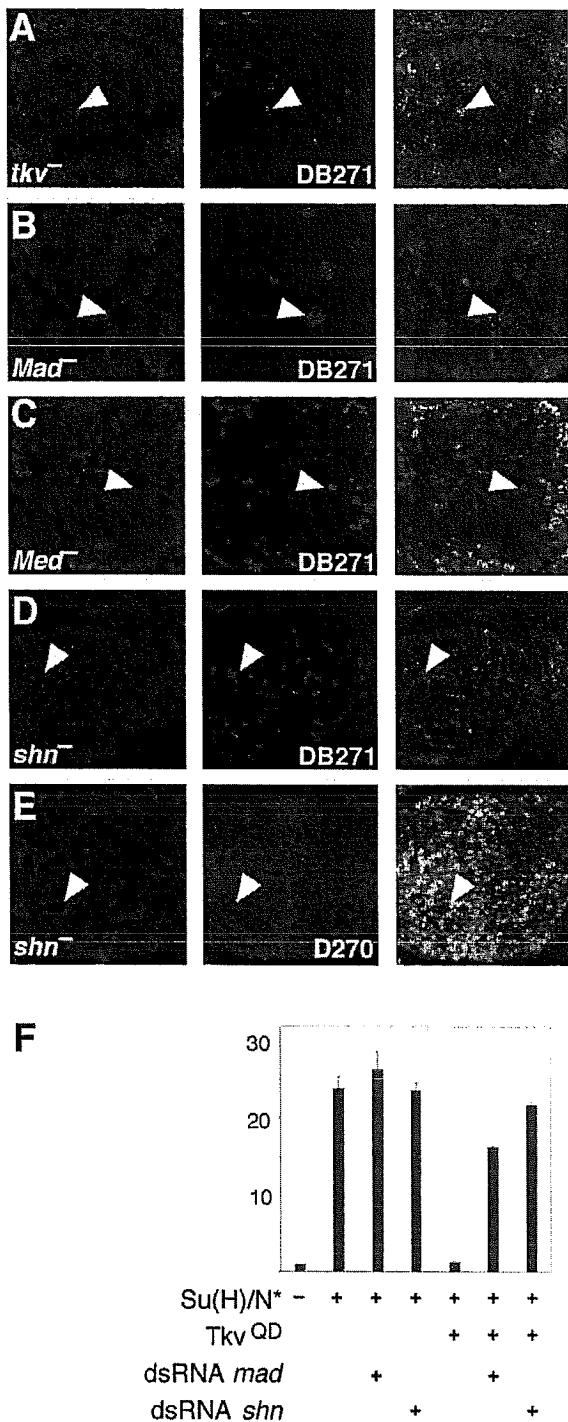


Figure 6. *brk* Silencer Activity Depends on Tkv, Mad, Med, and Shn Function

(A–D) Expression of reporter DB271 (described in Figure 4A) in wing discs with *tkv*, *Mad*, *Med*, and *shn* mutant clones. The left shows the expression of the marker gene (green), the loss of which indicates mutant genotypes. In the middle, the β -galactosidase expression of DB271 is visualized (red). A merge of both images is shown to the right. Expression of DB271 is strongly upregulated in medial *tkv*, *Mad*, and *Med*, as well as in *shn* mutant clones.

(E) In contrast, expression of D270 is not affected by these genotypes. D270 lacks *brk* silencer elements and is expressed ubiqui-

of Mad and Med was not retarded in its mobility by concomitant expression of the ShnCT variant lacking the three clustered zinc fingers (lane 9).

To investigate the molecular composition of the low mobility protein/DNA complexes, we cotransfected *Tkv*^{QD}, *Flag*⁺*Mad*, *Myc*⁺*Med*, and *V5*⁺*ShnCT* and assayed for the presence of the Flag, Myc, or V5 epitope tags by supershift analysis upon addition of the appropriate antibodies. In the absence of ShnCT, the complex contained both Mad and Med proteins as evidenced by supershifts with both the anti-Flag and the anti-Myc antibodies (Figure 7D). In the presence of *V5*⁺*ShnCT*, the low mobility complex was additionally supershifted by antibodies directed against the V5 epitope. However, when the same low mobility complex was produced with an untagged version of ShnCT, no increase in mobility was observed upon addition of the anti-V5 antibody, confirming the specificity of the assay (data not shown).

From these biochemical experiments, we conclude that Mad, Med, and Shn form a protein complex on the *brk* silencer element. Nuclear translocation of Mad and Med requires the activation of the Dpp signaling pathway (Raftery and Sutherland, 1999). Since Shn is only recruited to the *brk* silencer element in the presence of Mad and Med, it can be inferred that the complex can only be established in response to Dpp signaling. Moreover, our finding that both transcriptional repression as well as complex formation critically depend on the presence of the C-terminal three zinc finger motifs supports the notion that the *brk* silencer element controls *brk* expression by assembling a Mad/Med/Shn multiprotein complex.

Discussion

Dpp's ability to organize cellular patterns serves as a paradigm for the existence and mode of action of extracellular morphogen gradients. Most notably, Dpp gradients control cell fates along the dorsoventral axis of the early embryo and along the anteroposterior axis of imaginal discs (reviewed by Podos and Ferguson, 1999). In addition to its capacity to act at long range, Dpp elicits distinct outputs at different concentrations (Nellen et al., 1996; Lecuit et al., 1996; Ferguson and Anderson, 1992). BMP activity gradients have also been implicated in the control of vertebrate body pattern, particularly in the establishment of the dorsoventral axes of the early mesoderm, neural tube, and retina (Holley and Ferguson, 1997; Lee and Jessell, 1999; Sakuta et al., 2001). Major interest is devoted, therefore, to the mechanisms

tously in the wing pouch. Representative for the *tkv*, *Mad*, *Med*, and *shn*, only the results for *shn* mutant clones are shown. Arrowheads point to exemplary clones.

(F) *Drosophila* S2 cells were cotransfected with a reporter plasmid containing the *brk* S element fused to the Su(H)-response element, expression plasmids and dsRNA fragments as indicated below the panel. *Tkv*^{QD}-mediated repression is blocked when endogenous Mad or Shn are "knocked down" by RNAi. β -galactosidase levels are shown as X-fold activation over the basal activity of the reporter plasmid when cotransfected with the empty expression vector. dsRNA fragments are derived from the *Mad* (nucleotides 658–1230) or the *shn* (nucleotides 5011–5531) coding regions.

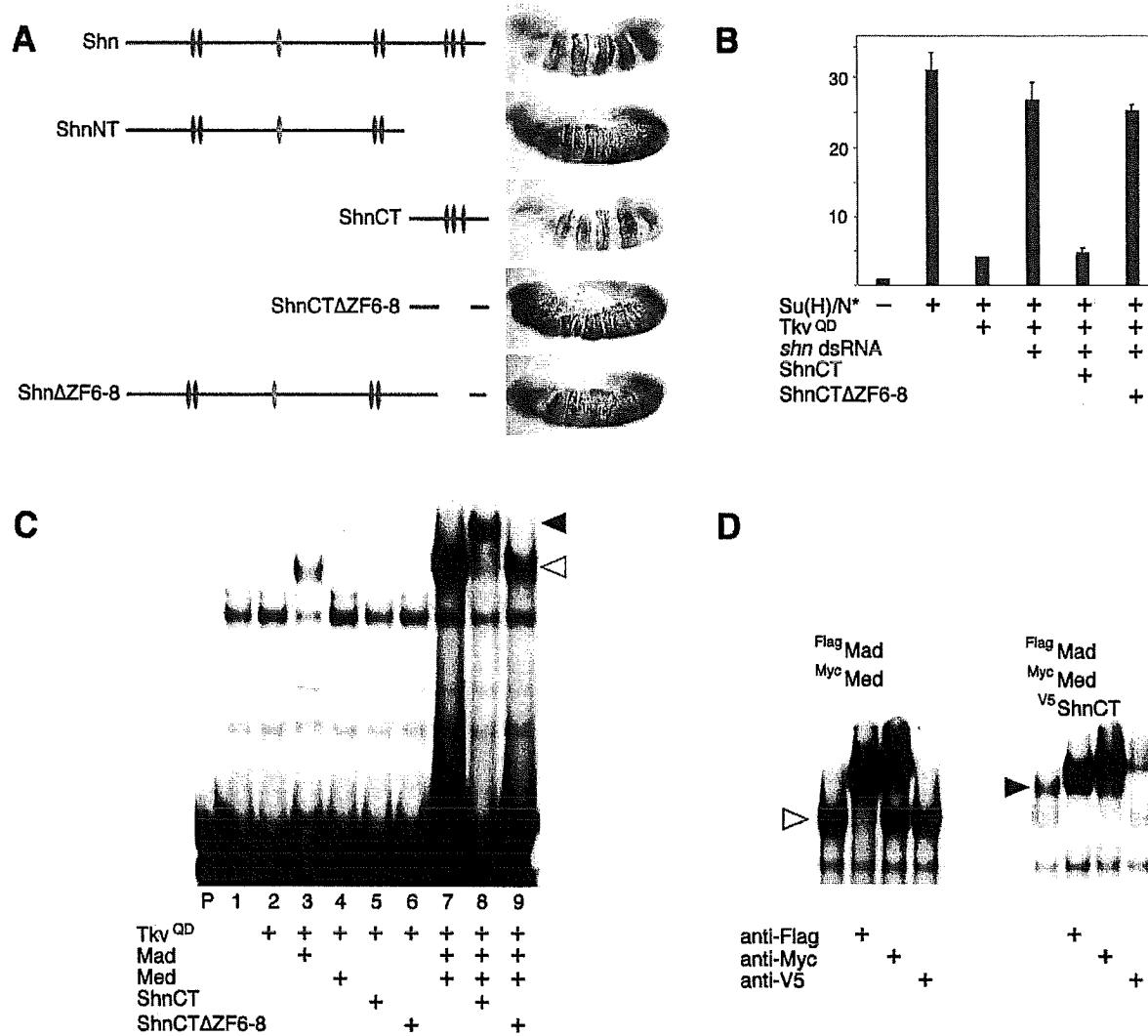


Figure 7. The Carboxy-Terminal Part of Shn Is Both Essential and Sufficient for Dpp-Dependent Repression In Vivo and in Cultured Cells and Forms a Complex with the *brk* Silencer, Mad, and Med

(A) Schematic representations of the Shn derivatives tested are shown to the left (blue ovals indicate zinc finger of the C2H2-type, light blue of the C2HC-type). Embryos transgenic for the illustrated *UAS-shn* constructs were tested for their ability to repress the expression of B14 or *brk* in vivo. The *UAS-shn* constructs were expressed in a *shn*^{TS5} mutant background together with *UAS-dpp* using a *paired-Gal4* driver. β -galactosidase expression of the B14 reporter is shown in stage 14/15 embryos to the right (assay described in Marty et al., 2000). The same results were obtained when these genotypes were assayed for *brk* transcript levels by *in situ* hybridization (data not shown). Shn, full-length (1–2529); ShnNT, N-terminal portion (1–1888); ShnCT, C-terminal portion (1888–2529); ShnCT Δ ZF6-8, C-terminal portion lacking zinc fingers 6 to 8 (i.e., residues 2263–2352); Shn Δ ZF6-8, full-length protein lacking zinc fingers 6 to 8 (i.e., residues 2263–2352). All Shn-fragments contained a Flag-epitope and expression of the tagged proteins was verified by antibody stainings of embryos (data not shown).

(B) S2 cell reporter gene assays. Cells were transfected with the plasmids indicated in combination with *shn* dsRNA to downregulate the expression of the endogenous Shn protein. The loss of Tkv^{QD}-mediated repression caused by *shn* RNAi can be restored by coexpression of ShnCT, but not ShnCT Δ ZF6-8. Note that the dsRNA used does not affect expression of the transfected carboxy-terminal Shn fragments, since it is derived from an upstream part of the *shn* coding region (corresponding to amino acids 1670–1842). Expression of the Shn construct has no effect on the Notch response as judged by cotransfections with the reporter plasmid containing the Su(H)-response element (data not shown).

(C) Lysates of S2 cells transfected with the indicated plasmids were analyzed in band shift assays using labeled *brk* fragment C as a probe. Transfection of Mad and Med leads to the formation of a protein/DNA complex of slow mobility (lane 7, indicated by the open arrowhead), which can be further retarded by cotransferring ShnCT (lane 8, filled arrowhead), but not ShnCT Δ ZF6-8 (lane 9). Note that in single transfections, only Mad has the ability to form a complex with *brk* S (lanes 3–6). Radiolabeled probe S was loaded alone (lane P) and after incubation with extract from untransfected cells (lane 1). The Shn/Mad/Med complex can also be observed in transfection experiments in which no Tkv^{QD} is expressed (data not shown), suggesting that phosphorylation of Mad is not a prerequisite for complex formation in vitro.

(D) Lysates from cells expressing ^{Flag}Mad, ^{Myc}Med without ^{v5}ShnCT (left), or with ^{v5}ShnCT (right) in combination with Tkv^{QD} were subjected to band shift assays in the presence of the indicated antibodies. Positions of the Mad/Med-complex (open arrowheads) and Mad/Med/ShnCT-complex (closed arrowheads) are indicated. In the absence of ShnCT, the complex contained both Mad and Med proteins as evidenced by supershifts with both the anti-Flag and the anti-Myc antibodies (Figure 7D, left). In the presence of ^{v5}ShnCT, the low mobility complex was supershifted by antibodies directed against the V5 epitope.

by which Dpp/BMP signaling controls gene expression. Important advances have recently been made by the discovery that a significant aspect of Dpp target gene control involves the repressive action of Brk, whose expression itself is regulated by Dpp (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999; Marty et al., 2000). Here, we first confirm and extend these findings by showing that the Dpp signaling system shapes an inverse profile of Brk expression, which serves as a mold for casting the spatial domains of Dpp target genes. Thus, the question of how the Dpp morphogen gradient is converted into transcriptional outputs can be largely reduced to the question of how Dpp generates an inverse transcriptional gradient of *brk* expression. We applied an unbiased approach to this problem by isolating the regulatory elements of *brk*. We then identify and characterize a protein complex that binds to and regulates the activity of these elements in a Dpp dose-dependent manner.

The Two Key Elements of *brk* Regulation

Dissection of the *brk* locus revealed two separable elements with opposite properties: a constitutive enhancer and a morphogen-regulated silencer. Both elements have a direct effect on the level of *brk* expression, and it is the net sum of their opposing forces that dictates the transcriptional activity of *brk* in any given cell. In this sense, expression of the *brk* gene behaves like a spring that is compressed by Dpp signaling. Its silencer and enhancer embody the variable compressing and constant restoring forces, respectively. As stated by Hooke's law, an increased elastic constant (e.g., two copies of the constitutive enhancer) either shifts the *brk* levels toward those normally present at more lateral positions or necessitates a correspondingly higher compressing force (e.g., more silencer elements or higher levels of Dpp signaling). Given the central role Brk plays in controlling growth and pattern together with the direct impact of the two regulatory elements on *brk* levels, it appears inevitable that their quantitative properties must exhibit a fine-tuned evolutionary relationship with each other and with those of the Dpp transduction system. It appears, furthermore, that both the *brk* enhancer as well as the *brk* silencer elements represent ideal substrates for evolutionary changes in morphology.

The Molecular Events at the *brk* Silencer

Based on our combined genetic and biochemical analysis, we propose that upon Dpp signaling the following key players meet at the *brk* silencer elements to execute repression: the Smad proteins Mad and Med and the zinc finger protein Shn. The role of Shn must be to direct the signaling input provided by Mad and Med into transcriptional silencing. In principle, two scenarios can be envisaged by which Shn fulfills this task. Shn could possess repressor activity (presumably via recruitment of corepressors) but lack the ability to bind the *brk* silencer and, hence, depend on Mad/Med for being targeted to its site of action. Alternatively, Shn could be prebound to the silencer, but only be capable of recruiting corepressors upon interaction with Mad/Med. Based on our observation that a Shn/DNA complex cannot be detected in the absence of Mad/Med, we favor the first

of these two possibilities. The molecular architecture of the protein complex binding to the *brk* silencer as well as the DNA sequences providing the specificity for the local setup of this complex remain to be determined in detail.

An additional protein, which appears to influence the events at the *brk* silencer, is Brk itself. Genetic experiments indicate that Brk negatively modulates its own expression, forming a short regulatory loop that contributes to the final shape of the Brk gradient (Hasson et al., 2001). This autoregulatory action occurs also via the *brk* silencer element (B.M., unpublished data), suggesting that Brk directly participates in the protein-protein or protein-DNA interactions at this site.

Most regulatory events ascribed to Smad proteins to date concern signaling-induced activation of target gene transcription. In the case of the *brk* silencer Shn could be regarded as a "switch factor" that converts an inherently activating property of Smad proteins into transcriptional repression activity. Indeed, it has been shown that Smad proteins have the ability to recruit general coactivators with histone acetyl transferase activity (reviewed by Massague and Wotton, 2000). However, in an alternative and more general view, Smad proteins per se may provide no bias toward activation or repression. Their main function may be to assemble transcriptional regulatory complexes involving other DNA binding proteins and endow these complexes with additional DNA binding capacity. Such associated DNA binding factors would not only determine target site specificity, but, by their recruitment of either coactivator or corepressor proteins, also define the kind of regulatory influence exerted on nearby promoters (Chen et al., 2002). Since Shn directs Mad/Med activity toward repression, we hypothesize the existence of at least one other such Mad/Med partner in *Drosophila* to account for Mad/Med-mediated activation of gene expression. Such Mad/Med-mediated activation appears to be required for peak levels of *sal* and *vg* transcription (Marty et al., 2000; Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a), as well as for defining gene expression patterns in domains where *brk* expression is completely repressed, e.g., close to the Dpp source of the dorsal embryonic ectoderm (Ashe et al., 2000; Jazwinska et al., 1999b).

The Specificity of Signaling-Regulated Repression

At the heart of our model is the direct causal relationship between the formation of a Shn/Mad/Med/*brk*-silencer complex and the silencing of *brk* gene transcription. Although the two observations have been derived from different experimental data sets (biochemical versus genetic, respectively), there is a firm correlation between the requirements for either event to occur. *brk* is not repressed when either (1) the *brk* silencer elements are lacking or mutated, when (2) Dpp input is prevented (and hence Mad is neither phosphorylated, nor nuclearly localized, nor associated with Med), or when (3) Shn is not present or is deprived of its C-terminal zinc fingers. The same set of requirements was observed for the formation of the Shn/Mad/Med/*brk* complex. Moreover, it is the concurrence of all three of these conditions that appears to provide the exquisite specificity to the Dpp-

regulated silencing of gene transcription. (1) It only occurs in conjunction with a functional *brk* silencer, or an equivalent element. (2) There is an absolute requirement for Dpp input in Shn-mediated silencing. Not even a partial repressor activity of Shn was observed in cells that do not receive Dpp signal (e.g., loss of *shn* function in cells situated in lateral-most positions of the wing disc does not cause a further upregulation of *brk* transcription). (3) Shn represents only one of several zinc finger proteins expressed in Dpp receiving cells, yet none of the other proteins is able to substitute for Dpp-mediated repression. A major determinant for the specificity with which Shn engages in the signaling-dependent protein/DNA complex appears to be the triple zinc-finger motif. Although it is likely that this structural feature is required for contacting specific nucleotides on the *brk* silencer, we can currently not exclude the possibility that some of the zinc fingers mediate protein-protein interactions between Shn and Mad, Med or other cofactors.

While all of the above-discussed elements contribute to the specificity of signaling-regulated repression, it is important to emphasize that one possibility for specificity has not been exploited. The *brk* repression element does not specifically impinge upon the constitutive *brk* enhancer but promiscuously diminishes transcriptional activation by heterologous enhancers. It is likely, therefore, that the *brk* repression element interferes directly with events at the promoter, a property that may permit it to function as a bona fide silencer.

From an Extracellular Gradient to a Nuclear Gradient to Growth and Thresholds

A fundamental characteristic of any morphogen system is that cells at different positions in the concentration gradient respond in qualitatively different ways. Cells must be able to activate different sets of genes at different threshold concentrations. The simplest way by which cells could produce two distinct responses at different threshold concentrations would be the employment of two kinds of receptors of different affinity for the morphogen. This mechanism does not appear to apply for the Dpp morphogen gradient, where Tkv and Punt appear to mediate both low- and high-threshold responses (see Gurdon et al., 1998). Thresholds could also be imposed at any downstream event in the signal transduction cascade. To our surprise, it appears that in the case of the Dpp morphogen, no such gates are in place, and the transcription of the *brk* gene is a negative image of the Dpp gradient. Thus, while our findings provide mechanistic insights into how an extracellular protein gradient is converted into a nuclear gradient of gene activity, they pass the burden of generating threshold effects on to downstream events. Several morphogen gradients operating in the early syncytial embryo, however, have been sufficiently well studied to explain the mechanistic principles of how a gradient of transcriptional activity can specify thresholds of gene activity and tissue differentiation (Struhl et al., 1989; Driever et al., 1989; Struhl et al., 1992; Jiang and Levine, 1993; Hoch and Jäckle, 1993).

A key difference between such embryonic transcriptional gradients and that of *brk* concerns the nature of

their outputs: while all of them affect cellular patterns, Brk also controls growth. Flattening the *brk* gradient during development has catastrophic effects: reducing its high end causes overgrowth (Campbell and Tomlinson, 1999), and increasing its low end causes growth arrest (Jaźwińska et al., 1999a; B.M., unpublished data). It may be this fundamental role in growth control that prohibits a discontinuous conversion of the Dpp morphogen gradient into its first transcriptional output. The identification of the elusive growth target(s) controlled by the Brk gradient represents one of the major challenges in the field.

Experimental Procedures

Reporter Transgenes

Inserts of reporter constructs B6 to B38 are derived from genomic lambda phages (G5 and G17, gifts from G. Campbell) and were subcloned into the P element reporter plasmid pX27 (Ségalat et al., 1994). Inserts of B71 to B220 were obtained by PCR, using B14 as a template. Constructs B255 and B261 consist of B216 plus a PCR fragment representing C (B255) or part of C (B261). Constructs B261 to B413 consist of B216 plus a double-stranded oligonucleotide derived from C. The sequence of the C subfragment S is as follows (from distal to proximal): AGTGTCTGGCGCGTAGCAAGACTGGC GACATTCTGCTGGTGGCGATCGCC. B413 contains a mutated form of S (mutated bases in lower case): AGTGTCTGGCGCGTAG CAAGACTGGCGACATTCTtaTGTTGGCGATCGCC. The insert of D270 is a chimera of constructs 10ΔG and 10-En-mut as shown in Figure 2 of Müller and Basler (2000). For construct DB271, fragment C was inserted at the 5' position of the *hsp70* promoter of D270. The *eve*-stripe-2 enhancer is represented by the MSE construct as published in Small et al. (1992). To obtain EB429, four copies of S were cloned into the EcoRI site of the MSE construct.

Marked Clones of Mutant Cells

Clones of mutant cells were generated by Flp-mediated mitotic recombination, subjecting late second or early third instar larvae to a 35°C heat-shock for 30 min. All mutant alleles used are molecular nulls. Genotypes of dissected larvae were as follows. *Mad* mutant clones: y w *hsp70*-flp; *Mad*[12] FRT40/ubi-nlsGFP FRT40; DB271. *Med* mutant clones: y w *hsp70*-flp; FRT82 e *Med*[1]/FRT82 2xhsp70-*mryc*; DB271. *tkv* mutant clones: y w *hsp70*-flp; *tkv*[a12] FRT40/ubi-GFP FRT40; DB271. *shn* mutant clones: y w *hsp70*-flp; FRT42 *shn*[TD5]/FRT42 *hsp70*-GFP; D270 or DB271. *tub*>*brk* clones: y w *omb-lacZ* *hsp70*-flp; *tub*>*CD2*, y+>*brk* and y w *hsp70*-flp; *CyO*[*sal-lacZ*], *tub*>*CD2*, y+>*brk*.

Immunohistochemistry

Imaginal discs from third instar larvae were fixed and stained by standard techniques. Antibodies were rabbit polyclonal anti-β-Gal (Cappel), mouse anti-cMyc (1-9E10.2, Santa Cruz Biotechnology), anti-rabbit 594 Alexa and anti-mouse 488 Alexa fluorescent secondary antibodies (Molecular Probes). To detect β-galactosidase activity, third instar larval discs were fixed and subjected to a standard X-gal color reaction for 2 hr at 37°C. For all X-gal stainings shown in this study, at least four independent transgenic lines were analyzed at standardized reaction conditions (2 hr at 37°C), and a representative disc was chosen for presentation.

S2 Cell Plasmids

S2 cell reporter plasmids containing the *brk* silencer were generated by inserting 100 bp of the 3' end of fragment C (comprising subfragment S) between the EcoRI and Asp718 sites in *hsplacCasper* and *4xSuh-lacZ* (Kirkpatrick et al., 2001). Epitope-tagged versions of *Tkv*^{op}, *Med*, *Shn*, *CT*, and *ShnCTΔZ* were cloned in the vector pAC5.1B/V5His (Invitrogen) for constitutive expression under the control of the *actin5c* promoter. Plasmids for constitutive expression of *Su(H)* and activated Notch were a gift from A. Laughon. dsRNA fragments were generated corresponding to nucleotides 658–1230

and 5011-5531 of the *Mad* and *shn* open reading frames, respectively.

Transfections and Reporter Gene Assays

For reporter gene assays 1.5×10^6 S2 cells were transfected with a total of 200 ng of DNA using the Effectene Transfection Reagent (Qiagen) (20 ng reporter plasmid, 5 ng of a plasmid constitutively expressing firefly luciferase, the indicated amount of expression plasmids and pAc5.1B/V5His to bring total DNA to 200 ng). For RNAi experiments 50 ng of the appropriate dsRNA fragment were cotransfected. Cells were lysed 48 hr after transfection for β -galactosidase and luciferase assays.

Band Shift Assays

The ^{32}P -labeled oligonucleotide probe (corresponding to silencer fragment S, see above) was generated by annealing and filling in overlapping oligonucleotides in the presence of $[\alpha-^{32}\text{P}]$ ATP. Epitope-tagged proteins were expressed in *Drosophila* S2 cells transfected with 100 ng of each expression plasmid. After 48 hr, cells were lysed in 100 μl of 100 mM TrisHCl (pH 7.8), 0.5% Triton X-100, 1 mM DTT, and protease inhibitors. For mobility shifts, 30 μg of protein was mixed with 10,000–20,000 cpm of probe in Binding Buffer (5 \times : 25 mM Tris-HCl [pH 7.6], 30% glycerol, 400 mM KCl, 50 mM MgCl₂, 50 μM ZnCl₂, 0.25% NP-40). Binding was allowed to proceed for 30 min on ice. Protein-DNA complexes were separated from free probe on 4% nondenaturing polyacrylamide gels (at room temperature for 130 min at 160V in 0.5 \times TBE). For supershifts, the following antibodies were added to the binding reaction: 20 ng of monoclonal anti-Flag (M2, Sigma), 8 ng of monoclonal anti-Myc (9B11, Cell Signaling), 0.5 μg monoclonal anti-V5 (Invitrogen).

Acknowledgments

We thank G. Campbell for providing genomic *brk* DNA and sharing unpublished results; S.B. Carroll and A. Laughon for plasmids; A. Jazwińska for *in situ* hybridizations; T. Marty, U. Nussbaumer, M.A. Vigano, and C. von Mering for advice and contributions in early stages of this project; M. Levine for discussions; and P. Gallant, R. Mann, and members of our laboratories for comments on the manuscript. This project was supported by the Roche Research Foundation, the Swiss National Science Foundation and the Cantons of Zürich and Basel.

Received: January 21, 2003

Revised: March 5, 2003

Accepted: March 10, 2003

Published: April 17, 2003

References

Affolter, M., Marty, T., Vigano, M.A., and Jaźwińska, A. (2001). Nuclear interpretation of Dpp signaling in *Drosophila*. *EMBO J.* **20**, 3298–3305.

Arora, K., Dai, H., Kazuko, S.G., Jamal, J., O'Connor, M.B., Letsou, A., and Warrior, R. (1995). The *Drosophila* *schnurri* gene acts in the Dpp/TGF-beta signaling pathway and encodes a transcription factor homologous to the human MBP family. *Cell* **81**, 781–790.

Ashe, H.L., Mannervik, M., and Levine, M. (2000). Dpp signaling thresholds in the dorsal ectoderm of the *Drosophila* embryo. *Development* **127**, 3305–3312.

Attisano, L., and Wrana, J.L. (2002). Signal transduction by the TGF-beta superfamily. *Science* **296**, 1646–1647.

Campbell, G., and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by brinker. *Cell* **96**, 553–562.

Chen, C.R., Kang, Y., Siegel, P.M., and Massagué, J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGF β receptor to c-myc repression. *Cell* **110**, 19–32.

Driever, W., Thoma, G., and Nüsslein-Volhard, C. (1989). Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the bicoid morphogen. *Nature* **340**, 363–367.

Entchev, E.V., Schwabedissen, A., and González-Gaitán, M. (2000). Gradient formation of the TGF-beta homolog Dpp. *Cell* **103**, 981–991.

Ferguson, E.L., and Anderson, K.V. (1992). Decapentaplegic acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. *Cell* **71**, 451–461.

Grieder, N.C., Nellen, D., Burke, R., Basler, K., and Affolter, M. (1995). *Schnurri* is required for *Drosophila* Dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. *Cell* **81**, 791–800.

Grimm, S., and Pflugfelder, G.O. (1996). Control of the gene optomotor-blind in *Drosophila* wing development by decapentaplegic and wingless. *Science* **271**, 1601–1604.

Gurdon, J.B., and Bourillot, P.Y. (2001). Morphogen gradient interpretation. *Nature* **413**, 797–803.

Gurdon, J.B., Dyson, S., and St Johnston, D. (1998). Cells' perception of position in a concentration gradient. *Cell* **95**, 159–162.

Hasson, P., Müller, B., Basler, K., and Paroush, Z. (2001). Brinker requires two corepressors for maximal and versatile repression in Dpp signalling. *EMBO J.* **20**, 5725–5736.

Hoch, M., and Jäckle, H. (1993). Transcriptional regulation and spatial patterning in *Drosophila*. *Curr. Opin. Genet. Dev.* **3**, 566–573.

Holley, S.A., and Ferguson, E.L. (1997). Fish are like flies are like frogs: conservation of dorsal-ventral patterning mechanisms. *Bioessays* **19**, 281–284.

Hudson, J.B., Podos, S.D., Keith, K., Simpson, S.L., and Ferguson, E.L. (1998). The *Drosophila* *Medea* gene is required downstream of dpp and encodes a functional homolog of human Smad4. *Development* **125**, 1407–1420.

Inoue, H., Immura, T., Ishidou, Y., Takase, M., Udagawa, Y., Oka, Y., Tsuneizumi, K., Tabata, T., Miyazono, K., and Kawabata, M. (1998). Interplay of signal mediators of decapentaplegic (Dpp): molecular characterization of Mothers against dpp, Medea, and daughters against dpp. *Mol. Cell* **9**, 2145–2156.

Jaźwińska, A., Kirov, N., Wieschaus, E., Roth, S., and Rushlow, C. (1999a). The *Drosophila* gene brinker reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563–573.

Jaźwińska, A., Rushlow, C., and Roth, S. (1999b). The role of brinker in mediating the graded response to Dpp in early *Drosophila* embryos. *Development* **126**, 3323–3334.

Jiang, J., and Levine, M. (1993). Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the dorsal gradient morphogen. *Cell* **72**, 741–752.

Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., Magee, J., and Carroll, S.B. (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila* vestigial gene. *Nature* **382**, 133–138.

Kim, J., Johnson, K., Chen, H.J., Carroll, S., and Laughon, A. (1997). *Drosophila* Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature* **388**, 304–308.

Kirkpatrick, H., Johnson, K., and Laughon, A. (2001). Repression of dpp targets by binding of brinker to Mad sites. *J. Biol. Chem.* **276**, 18216–18222.

Lecuit, T., Brook, W.J., Ng, M., Calleja, M., Sun, H., and Cohen, S.M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* **381**, 387–393.

Lee, K.J., and Jessell, T.M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. *Annu. Rev. Neurosci.* **22**, 261–294.

Marty, T., Müller, B., Basler, K., and Affolter, M. (2000). *Schnurri* mediates Dpp-dependent repression of brinker transcription. *Nat. Cell Biol.* **2**, 745–749.

Massagué, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753–791.

Massagué, J., and Wotton, D. (2000). Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J.* **19**, 1745–1754.

Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H., and Tabata, T. (1999). brinker is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes. *Nature* **398**, 242–246.

Morgan, T.H. (1897). Regeneration in *Allotlobophora foetida*. *Roux's Arch Dev. Biol.* *5*, 570–586.

Müller, B., and Basler, K. (2000). The repressor and activator forms of *Cubitus interruptus* control Hedgehog target genes through common generic Gli-binding sites. *Development* *127*, 2999–3007.

Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996). Direct and long-range action of a Dpp morphogen gradient. *Cell* *85*, 357–368.

Podos, S.D., and Ferguson, E.L. (1999). Morphogen gradients: new insights from Dpp. *Trends Genet.* *15*, 396–402.

Raftery, L.A., and Sutherland, D.J. (1999). TGF- β family signal transduction in *Drosophila* development: from Mad to Smads. *Dev. Biol.* *210*, 251–268.

Ramírez-Weber, F.A., and Kornberg, T.B. (1999). Cytonemes: cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. *Cell* *97*, 599–607.

Sakuta, H., Suzuki, R., Takahashi, H., Kato, A., Shintani, T., Iemura, S., Yamamoto, T.S., Ueno, N., and Noda, M. (2001). Ventroptin: a BMP-4 antagonist expressed in a double-gradient pattern in the retina. *Science* *293*, 111–115.

Ségalat, L., Berger, G., and Lepesant, J.A. (1994). Dissection of the *Drosophila* pourquoi-pas? promoter: complex ovarian expression is driven by distinct follicle cell- and germ line-specific enhancers. *Mech. Dev.* *47*, 241–251.

Small, S., Blair, A., and Levine, M. (1992). Regulation of even-skipped stripe 2 in the *Drosophila* embryo. *EMBO J.* *11*, 4047–4057.

Strigini, M., and Cohen, S.M. (1999). Formation of morphogen gradients in the *Drosophila* wing. *Semin. Cell Dev. Biol.* *10*, 335–344.

Struhl, G., Struhl, K., and Macdonald, P.M. (1989). The gradient morphogen bicoid is a concentration-dependent transcriptional activator. *Cell* *57*, 1259–1273.

Struhl, G., Johnston, P., and Lawrence, P.A. (1992). Control of *Drosophila* body pattern by the hunchback morphogen gradient. *Cell* *69*, 237–249.

Sturtevant, M.A., Biehs, B., Marin, E., and Bier, E. (1997). The spalt gene links the A/P compartment boundary to a linear adult structure in the *Drosophila* wing. *Development* *124*, 21–32.

Tabata, T. (2001). Genetics of morphogen gradients. *Nat. Rev. Genet.* *2*, 620–630.

Tanimoto, H., Itoh, S., ten Dijke, P., and Tabata, T. (2000). Hedgehog creates a gradient of Dpp activity in *Drosophila* wing imaginal discs. *Mol. Cell* *5*, 59–71.

Telemán, A.A., and Cohen, S.M. (2000). Dpp gradient formation in the *Drosophila* wing imaginal disc. *Cell* *103*, 971–980.

Torres-Vazquez, J., Warrior, R., and Arora, K. (2000). Schnurri is required for Dpp-dependent patterning of the *Drosophila* wing. *Dev. Biol.* *227*, 388–402.

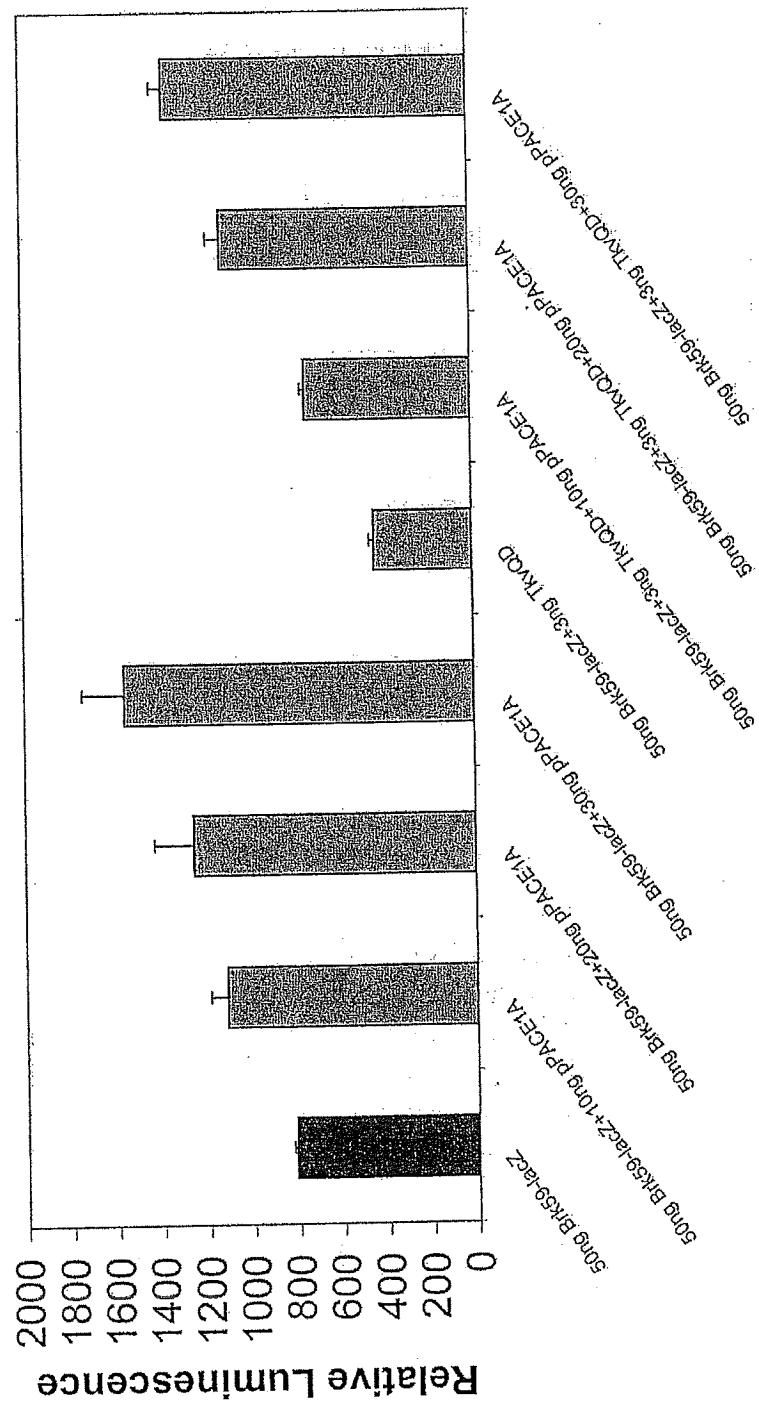
Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T.B., Christian, J.L., and Tabata, T. (1997). Daughters against dpp modulates dpp organizing activity in *Drosophila* wing development. *Nature* *389*, 627–631.

Turing, A.M. (1952). The chemical basis of morphogenesis. *Phil. Trans. Roy. Soc. Lond. B* *237*, 37–72.

Wisotzkey, R.G., Mehra, A., Sutherland, D.J., Dobens, L.L., Liu, X., Dohrmann, C., Attisano, L., and Raftery, L.A. (1998). Medea is a *Drosophila* Smad4 homolog that is differentially required to potentiate Dpp responses. *Development* *125*, 1433–1445.

Wolpert, L. (1989). Positional information revisited. *Development* *107 Suppl.* 3–12.

Exhibit B



A Novel Histone Deacetylase Inhibitor Identified by High-Throughput Transcriptional Screening of a Compound Library¹

Gloria H. Su, Taylor A. Sohn, Byungwoo Ryu, and Scott E. Kern²

Departments of Pathology [G. H. S.] and Surgery [T. A. S.] and The Oncology Center [G. H. S., B. R., S. E. K.J, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205-2196

Abstract

Libraries of compounds are increasingly becoming commercially available for the use of individual academic laboratories. A high-throughput system based on stably integrated transcriptional reporter was used to screen a library of random compounds to identify agents that conferred robust augmentation of a signal transduction pathway. A novel histone deacetylase (HDAC) inhibitor, termed scriptaid, conferred the greatest effect, a 12- to 18-fold augmentation. This facilitation of transcriptional events was generally applicable to exogenous gene constructs, including viral and cellular promoters, different cell lines and reporter genes, and stably integrated and transiently introduced sequences. Scriptaid did not interfere with a further induction provided by stimulation of the cognate signal transduction pathway (transforming growth factor β /Smad4), which implied the functional independence of ligand-stimulated transcriptional activation and histone acetylation states in this system. Additional insights into this and other signal transduction systems are likely to be afforded through the application of compound screening technologies.

Introduction

Many signal transduction pathways couple ligand-initiated events that affect the regulation of gene expression. The elaboration of signal transduction pathways is important in tumor biology because the mediators and regulators of signaling pathways can represent useful targets for diagnosis and therapy.

Currently, the common methods used to identify the mediator and regulators of a pathway involve physical interactions (two-hybrid analysis and protein affinity chromatography), experimental genetic means (mutagenesis of bacteria or yeast), and observations of natural genetic variance (human tumors and inherited disease susceptibility). Cloning by physical interactions is rather inefficient and laborious and can be biased by the initial choice of the bait. Experimental genetic methods are efficient and unbiased but difficult to apply to mammalian cells and human disease. Research based on natural genetic variance is highly relevant to the understanding of human disease, but the sample size of such an approach is often limited. The screening of random libraries of chemical compounds can encompass all of the benefits of current methodologies; they can be unbiased and high-throughput and can be used to probe and dissect complex biological systems in mammalian cells (1, 2). Screening of compound libraries can also directly produce candidates for therapeutic and experimental applications.

The "true" activation of a signaling or regulatory pathway can be difficult to measure. The detection of a downstream transcriptional

event using a specific reporter construct in cells is a common method. This type of manipulation of a pathway requires a basic understanding of the participating members of the pathway, but the understanding is often incomplete and imprecise. To test a promoter or an enhancer under adequate sensitivity, the selection of reporter genes often is limited. The interpretation of results using downstream reporter constructs can be misled by unanticipated positive or negative influences. That is, it is often difficult to distinguish release of inhibition from true activation, and *vice versa*. Furthermore, the system depends on the recruitment of the general transcriptional apparatus, the interaction of which with the system under study is seldom known in advance.

We chose to address these issues by the implementation of a high-throughput compound screening, using a stably integrated reporter construct to identify reliable and important regulators of a tumor-suppressive pathway. The TGF β ³ pathway is well studied biologically and comprises a number of human tumor-suppressor genes, including SMAD4 (MADH4, DPC4; Refs. 3–5). Our reporter construct contains Smad-binding elements (p6SBE-luc) and allows us to measure processes that result in the nuclear localization of Smad4 (6). The discovery of agents that would interact with or bypass deficits in the TGF β pathway by augmenting the action of downstream Smad4 would likely be useful to the biological understanding of this tumor-suppressive pathway.

Materials and Methods

Reporter Constructs. p6SBE-luc and p6MBE-luc were engineered by inserting six copies of the palindromic SBE or of the MBE (an inactive mutant version) behind the minimal SV40 promoter in the pGL3-promoter vector (Promega, Madison, WI; Ref. 6).

Cell Lines. PANC-1 and MDA-MB-468 cell lines were purchased from American Type Culture Collection (Manassas, VA). Stable transfectants were generated by cotransfection of pcDNA3.1 (Invitrogen, Carlsbad, CA) and p6SBE-luc into PANC-1 cells with Lipofectamine (Life Technologies, Inc.). Transfected cells were diluted and selected in multiple 96-well plates in the presence of 0.5 mg/ml of G418 (Life Technologies, Inc.). Single clones were expanded and tested for basal luciferase expression and TGF β inducibility. One clone was chosen on the basis of high (6- to 8-fold) induction of luciferase by 0.5 ng/ml TGF β (R&D Systems, Minneapolis, MN).

Compound Screening. Each compound of the library (DIVERSet, ChemBridge, San Diego, CA) was dissolved and diluted in DMSO at 1 mg/ml. Cells were plated in 96-well cluster plates (Corning, Cambridge, MA) and incubated with each compound, after further dilution in culture medium to the final concentration of 2 μ g/ml, for 16–18 h. Luciferase activity was measured on the addition of Steady-Glo substrate (Promega). Up to 16 96-well plates could be assembled in a Wallac Trilux photodetector (Wallac, Gaithersburg, MD) for measurement. All of the readouts from each experiment were compared with the control wells, and a number reflecting the relative increase in luciferase activity was calculated for each chemical by using Excel (Microsoft, Redmond, WA) spreadsheets.

³ The abbreviations used are: TGF, transforming growth factor; HDAC, histone deacetylase; TSA, trichostatin A; SBE, Smad-binding element; MBE, mutated SBE; β -gal, β -galactosidase; CMV, cytomegalovirus; CBP, CREB-binding protein.

Received 1/7/00; accepted 4/28/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by the NIH SPORE (Specialized Program of Research Excellence) in Gastrointestinal Cancer Grant CA 62924 and NIH Grant CA 68228.

² To whom requests for reprints should be addressed, at the Department of Oncology, 451 Cancer Research Building, 1650 Orleans Street, The Johns Hopkins University School of Medicine, Baltimore, MD 21231. Phone: (410) 614-3314; Fax: (410) 614-9705; E-mail: sk@jhmi.edu.

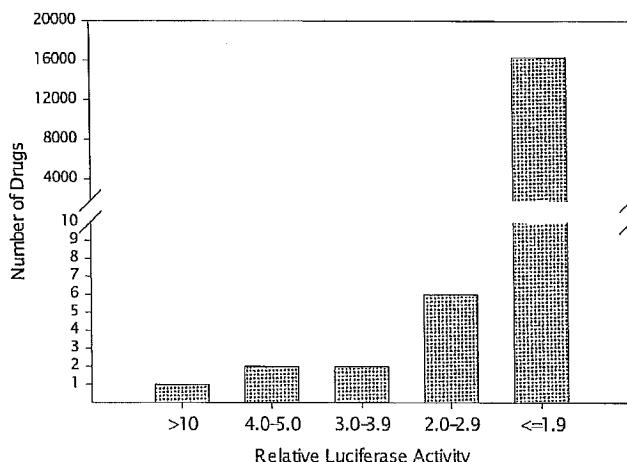


Fig. 1. The distribution of compounds in accordance to their relative luciferase activity. The entire library, consisting of 16,320 compounds, was screened with the p6SBE-luc reporter construct stably integrated in PANC-1 cells. The relative luciferase activity was calculated in comparison with untreated cells.

Immunoblotting Assay of Histone Acetylation. PANC-1 cells were treated with 2 μ g/ml of scriptaid (ChemBridge) or 0.1 to 0.32 μ g/ml of TSA (Sigma, St. Louis, MO) for 18 h in culture medium. Treated and untreated cells were harvested with trypsin-EDTA (Life Technologies, Inc.), washed with PBS (Life Technologies, Inc.), and resuspended in a protein sample buffer. Protein concentration was determined by BCA protein assay reagents (Pierce, Rockford, IL). Fifty μ g of proteins from each sample was loaded on a 12% denaturing polyacrylamide gel. Proteins were subsequently transferred to a nylon membrane (Imobilon P, Millipore, Burlington, MA) using Milliblot-Graphite Electrophoresis (Millipore). The nylon membrane was incubated with rabbit antihuman acetyl-lysine antibody (Upstate Biotechnology, Waltham, MA) at 1:1000 dilution, followed by goat antirabbit antibody coupled to horseradish peroxidase (Pierce) at 1:2000 dilution, developed with SuperSignal substrates (Pierce), and detected by film (BioMax, Kodak, Rochester, NY).

Survival Curve. Equal numbers of cells were plated in six-well plates in the absence or presence of scriptaid or TSA at different concentrations. After 18 h of incubation, cell numbers were determined by trypan blue exclusion. Percent survival of the treated cells was calculated in comparison to the untreated sample, which was considered to represent 100%.

Transfection Assay. Each transient transfection experiment was done in duplicate in six-well plates. Lipofectamine (Life Technologies, Inc.) was used as directed by the manufacturer. The DNA-lipofectamine mixture was removed from cells after 4–5 h of transfection, and culture media with or without compounds or TGF β was then added to the cells. Sixteen to 18 h from the start of the transfection, cell lysates were prepared with Reporter Lysis Buffer (Promega) for luciferase and β -gal assays. Luciferase was measured using The Luciferase Assay System (Promega) and β -gal assay was performed as described previously (6). Studies of the SV40 promoter included all of the experiments performed with p6SBE-luc, p6MBE-luc, and pGL3-control (Promega) plasmids. Studies of the CMV promoter were done using pCMV β (Clontech, Palo Alto, CA), and those of human ubiquitin c promoter were done using pUB6/V5-lacZ (Invitrogen).

Results

Identification of Scriptaid. The entire library, consisting of 16,320 compounds, was screened. Eleven compounds were associated with a 2- to 5-fold induction of luciferase activity, and one with a 12-fold activation (Fig. 1). Additional studies on the latter compound (Identification No. 217444; 6-(1,3-dioxo-1H,3H-benzo[*de*]isoquinolin-2-yl)-hexanoic acid hydroxyamide, we termed scriptaid) are reported here (Fig. 2). A related compound (Identification No. 158497; 4-(1,3-dioxo-1H,3H-benzo[*de*]isoquinolin-2-yl)-N-hydroxy-butyramide, we termed nullscript), which did not induce the p6SBE-luc reporter construct in the initial screen, was identified from the

library using ChemFinder (CambridgeSoft, Cambridge, MA) by its structural similarity to scriptaid (Fig. 2). The results were validated by repeated determinations in the screening assay and subsequently by a dose-response curve performed on PANC-1 cells that contained stably integrated p6SBE-luc (Fig. 3).

Scriptaid As a Novel Inhibitor of HDAC. Scriptaid was speculated to be a novel HDAC inhibitor because of its structural similarity to the class of hydroxamic acid-containing HDAC inhibitors, which

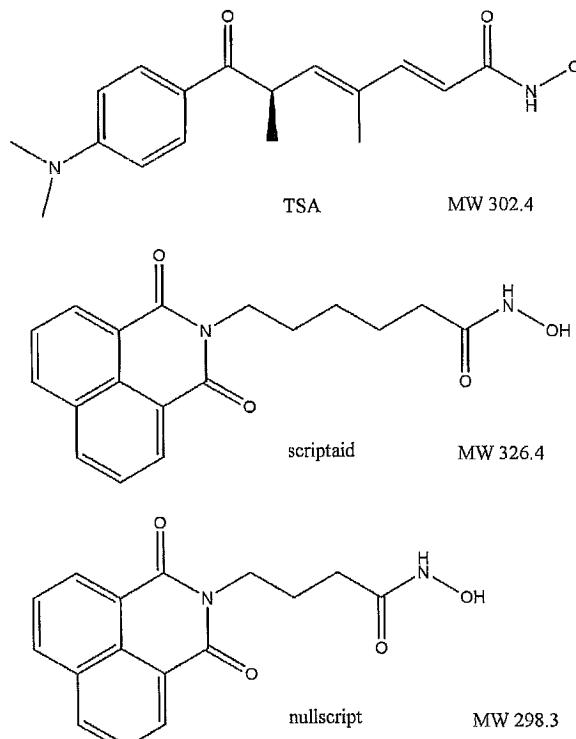


Fig. 2. Structural similarities of TSA, scriptaid, and nullscript. TSA, scriptaid, and nullscript possess the same hydroxamic acid group, an aliphatic chain, and an aromatic cap at the other end. The aliphatic linkers of TSA and scriptaid are 5-carbon in length, whereas the linker is only 3-carbon in nullscript.

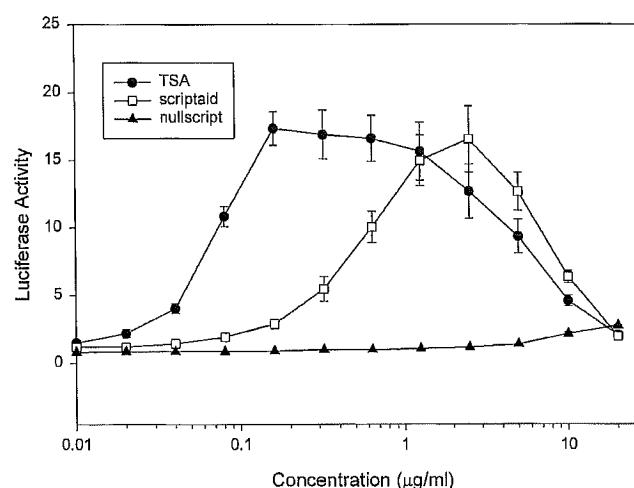


Fig. 3. Dose responses of TSA and scriptaid transcriptional facilitation in a stably transfected cell line. Luciferase activity was determined using PANC-1 cells having stably integrated p6SBE-luc at the indicated concentration of compounds. Data represent averages of two to three experiments and SE.

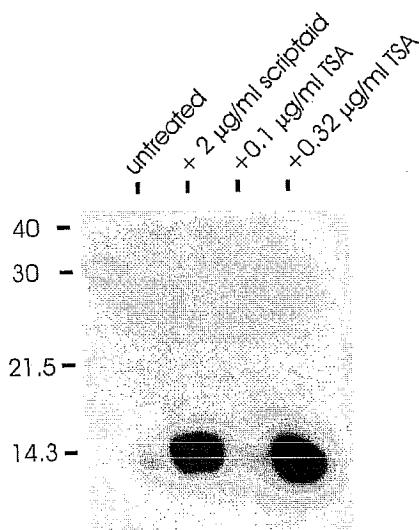


Fig. 4. HDAC inhibition by scriptaid. PANC-1 cells were untreated or treated with scriptaid (2 μ g/ml) or TSA (0.1 or 0.32 μ g/ml). Acetylated histones were detected by antihuman acetyl-lysine antibody immunoblot.

include TSA (Fig. 2). The direct interaction of TSA and a HDAC has been examined by crystallography (7). The hydroxamic acid group of TSA coordinates the zinc atom in the polar pocket of HDAC in the crystal structure of the HDAC-Zn²⁺-TSA complex. The hydroxamic acid group on TSA is attached to a five-carbon (excluding the carbon elements of the hydroxamic acid group or the keto group) aliphatic chain that spans a narrow tube-like pit formed by the surface of HDAC. The double bonds and the methyl-group in the aliphatic chain of TSA (Fig. 2) are not necessary for its inhibitory function (7). The bulky end-group on the opposite end of the aliphatic chain is positioned outside the entrance to the pit. Similar to TSA, scriptaid has a five-carbon linker between a bulky end-group and the hydroxamic acid moiety. Nullscript, which is almost identical to scriptaid except for a three-carbon (rather than five-carbon) linker (Fig. 2), was inactive in transcriptional facilitation at corresponding concentrations (Fig. 3), which confirmed a minimal requirement for the length of the linker chain expected for this class of HDAC inhibitors.

The use of scriptaid resulted in a >100-fold increase in histone acetylation (Fig. 4) in cultured cells, which confirmed scriptaid as a HDAC inhibitor.

Functional Comparison with TSA. To evaluate the potency of scriptaid, TSA was used as the reference compound in the following experiments. Optimal concentration was determined for both scriptaid and TSA (Fig. 3). Scriptaid worked optimally at 2–2.5 μ g/ml (6–8 μ M), and TSA activity peaked at 0.32 μ g/ml (1 μ M). At its optimal concentration for transcriptional facilitation, scriptaid was not lethal to one cell line and had limited effects (80% survival) on another (Fig. 5A). TSA was cytotoxic for two cell lines at its optimal concentration range (Fig. 5B). TSA at its minimal toxic concentration (0.1 μ g/ml; 85–90% survival) was less efficient at inhibiting endogenous histone deacetylation (Fig. 4).

Smad proteins can physically associate with the histone acetylases p300 or CBP, and it has been suggested that the TGF β /Smad4 signaling pathway might activate gene transcription through such a mechanism (8–10). It was, therefore, important to determine whether the manipulation of histone acetylation status would interfere with the results of an assay for relative transcriptional induction mediated by the SBE sequence. It has been shown previously that p6SBE-luc, but not p6MBE-luc, can be induced by TGF β treatment (6). pCMV β was,

therefore, cotransfected with p6SBE-luc or p6MBE-luc as a TGF β -insensitive control. The presence of scriptaid increased the transcription of all of the three reporters by twelve-fold. Using a normalization for (cotransfected) β -gal expression, the measured magnitude of the ability of TGF β to specifically induce p6SBE-luc remained the same (Fig. 6A), irrespective of the presence or absence of scriptaid. Scriptaid could thus facilitate transcription independent of a positive inducer of transcription, producing multiplicative rises in reporter activity. Similar effects were seen with either scriptaid or TSA when the reporter construct was stably integrated into the host cell genome (Fig. 6, B and C). Scriptaid and TSA can proportionally enhance the induction of an integrated p6SBE-luc construct without interfering with TGF β -stimulated transcriptional responses.

To further evaluate the range of promoter elements subject to scriptaid induction, additional reporters were used in transient transfections. Scriptaid was capable of inducing high expression of p6MBE-luc (Fig. 7A), pCMV β (Fig. 7B), and pUB6/V5-LacZ (Fig. 7C), driven by viral (SV40 and CMV) or human (ubiquitin c, UB6) promoters. This general facilitation of transcription by scriptaid did not depend on the specificity of the enhancer (SBE *versus* MBE), the type of promoter (viral *versus* cellular), the product of the reporter gene (luciferase *versus* β -gal), nor on the integration status of the

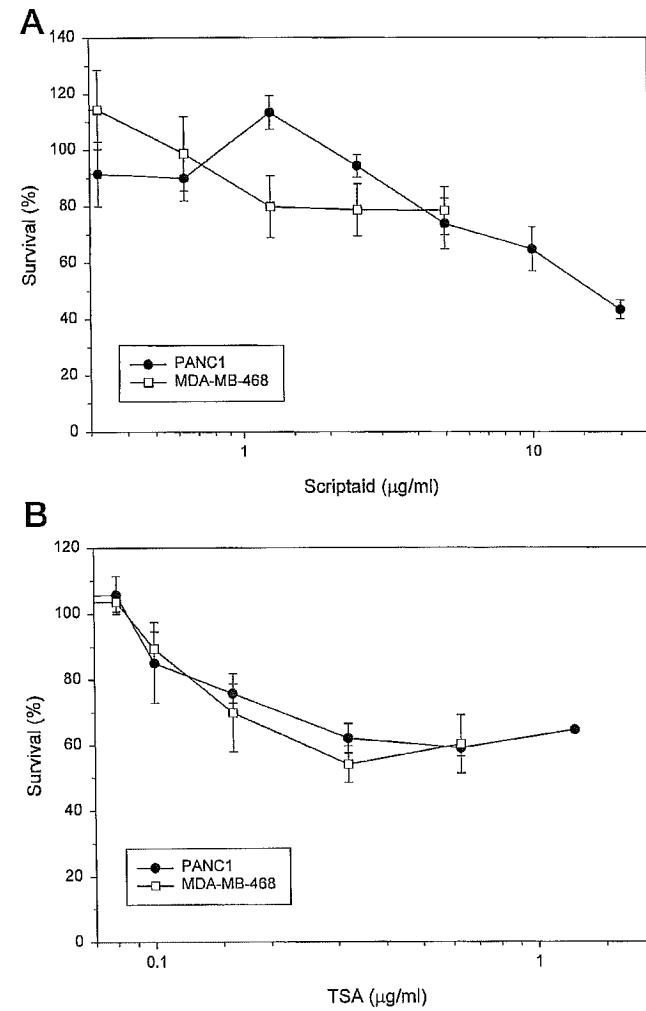


Fig. 5. Effects of compounds on cell survival. The survival of PANC-1 and MDA-MB-468 in the presence of scriptaid (A) and TSA (B) was determined by trypan blue exclusion after an 18-h incubation in the presence of compound. Data represent averages of two to four experiments and SE.

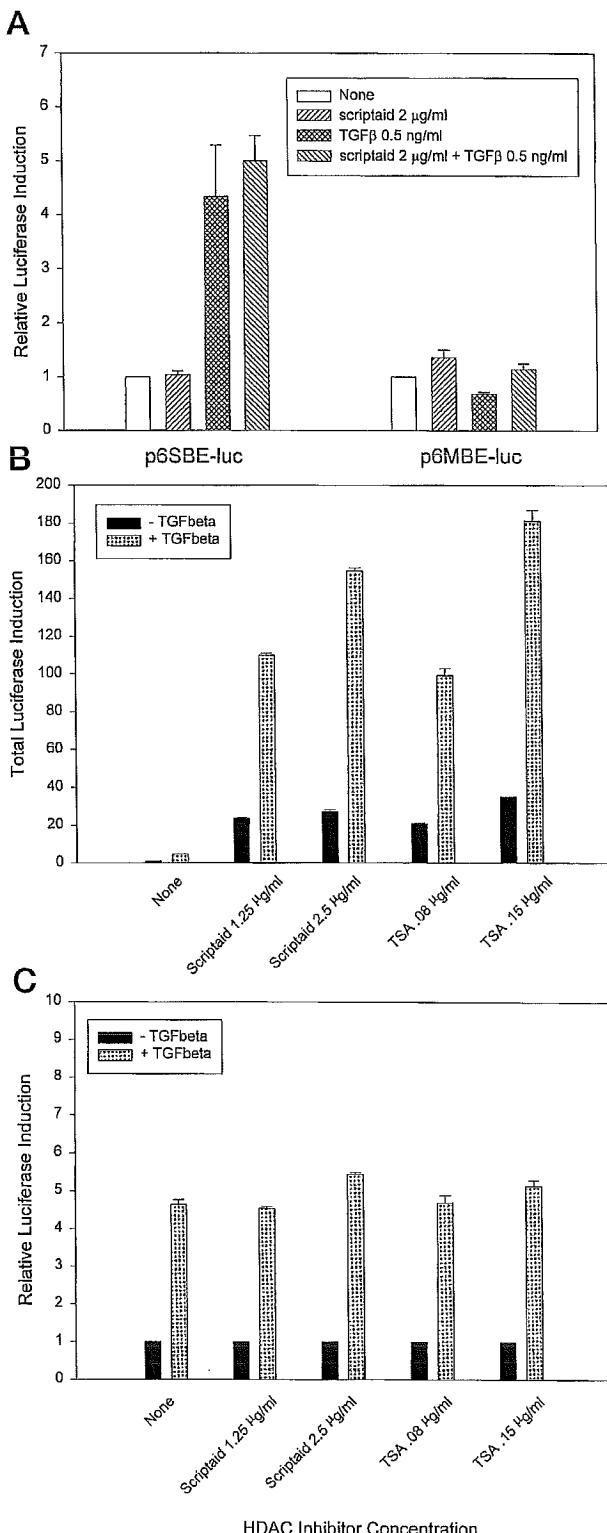


Fig. 6. Proportional effects of scriptaid and TSA on the TGF β /Smad4 signal transduction assay. *A*, p6SBE-luc or p6MBE-luc was cotransfected with pCMV- β into PANC-1 cells. Transfected cells were untreated or treated with TGF β or/and scriptaid for 18 h. Luciferase activities were proportionally enhanced in the presence of scriptaid. Relative luciferase induction was determined after normalization to the TGF β -noninducible pCMV- β control, itself subject to scriptaid facilitation (see Fig. 7). Data represent averages of two experiments and SE. *B* and *C*, PANC-1 cells containing the stably integrated p6SBE-luc were treated with scriptaid or TSA in the absence or presence of TGF β (1 ng/ml). Total luciferase induction is presented in *B*. Relative luciferase induction (*C*) was determined after normalization to the values observed with the HDAC inhibitor alone.

reporter construct (stable *versus* transient). The ability of scriptaid to facilitate transcriptional activation was consistently robust and concentration-dependent in both stable and transient reporter assays (Fig. 3, 7, and data not shown). In contrast, the performance of TSA was less predictable at its optimal concentration (Fig. 7, *A* and *B*; see *MDA-MB-468*). Lowering the concentration of TSA (0.1 μ g/ml) could mitigate this lack of consistency, but in doing so, the efficiency of TSA as an HDAC inhibitor or a general transcription facilitator was significantly compromised (Fig. 4 and 7).

Discussion

We successfully confirmed the feasibility of a high-throughput reporter system to efficiently screen a large compound library in mammalian cells. The screening identified several compounds capable of augmenting the reporter activity, and a specific mechanism for one of the compounds was defined. This confirmed the suggested utility of this application of compound screening in mammalian cells for the discovery of additional small compound interactors that could help characterize the components of this pathway.

Scriptaid is a novel HDAC inhibitor that belongs to an existing class of hydroxamic acid-containing HDAC inhibitors. Scriptaid possesses a general property of transcriptional facilitation that applies to stably integrated or transiently transfected exogenous constructs, to promoters derived from viruses or an endogenous gene, to multiple reporter genes, and to different cell lines. Scriptaid does not interfere with the ability of a reporter construct to measure the positive (purely inductive) activation of a transcription factor in response to a known signal transduction stimulus. In relation to other members of its class, the optimal concentration of scriptaid (6–8 μ M) is similar to those reported for suberoylanilide hydroxamic acid (2 μ M) and m-carboxy-cinnamic bis-hydroxamide (4 μ M; Ref. 11), higher than TSA (1 μ M, as measured here), and much lower than those reported for hexamethylenebisacetamide (5000 μ M) and diethyl bis-(penta-methylene-*N,N*-dimethylcarboxamide) malonate (400 μ M; Ref. 11). Our data suggested some advantages of scriptaid over TSA in the range of promoters subject to predictable effects (Fig. 4 and 7) and in cellular toxicity (Fig. 4 and 5), although some degree of cellular toxicity may be a general feature of this class of compounds when used at transcriptionally effective concentrations (11).

The ability of scriptaid to indiscriminately facilitate transcriptional activation and its facilitation of detection of a positive transcriptional signal suggest the usage of scriptaid as a useful reagent for transactivation assays in reporter systems, perhaps allowing the use of difficult-to-transfect cells, the use of the available but less sensitive reporter genes, such as green fluorescent protein, or the minimization of culture volumes to aid high-throughput compound or biological screening and for adaptation to robotic handling. A reduction in the signal transduction strength needed to detect the operation of a reporter suggests a utility in the measurement of signal transduction events at a lower and thus more physiological range. For example, the use of scriptaid would be expected to reduce the requirement for protein overexpression or for high (pharmacological) levels of ligand often used to facilitate the evaluation of a signaling pathway. Application to other protein expression methods is also possible.

Currently underappreciated is the strength of the background of transcriptional repression that acts on general-utility promoters. Use of a relative nontoxic HDAC inhibitor such as scriptaid, thus, could conceivably simplify the interpretation of transcriptional reporter assays. We observed that at least 90% of the potential magnitude of the inducible transcriptional activation of our reporter system was originally repressed. It is known that the expression of some genes is regulated by the degree of histone acetylation (12). Thus, a 2-fold

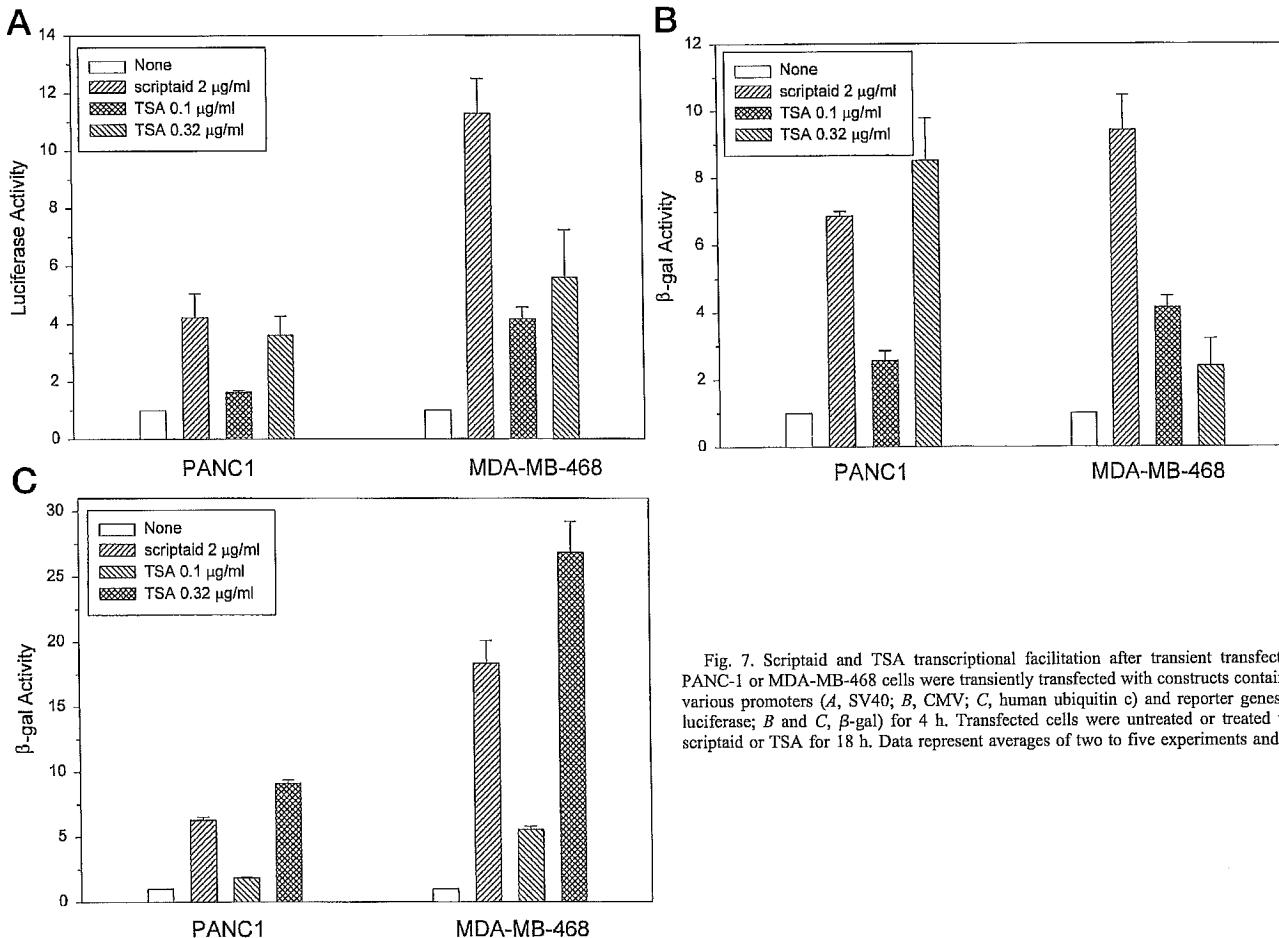


Fig. 7. Scriptaid and TSA transcriptional facilitation after transient transfection. PANC-1 or MDA-MB-468 cells were transiently transfected with constructs containing various promoters (A, SV40; B, CMV; C, human ubiquitin) and reporter genes (A, luciferase; B and C, β -gal) for 4 h. Transfected cells were untreated or treated with scriptaid or TSA for 18 h. Data represent averages of two to five experiments and SE.

induction seen in an experimental situation may not always represent a 100% increase in strength of transactivation *per se* but could be mimicked, for example, by a 10% decrease in repression. These two possibilities could presumably be distinguished by the use of scriptaid and other HDAC inhibitors. In some reporter systems, negative effects on transcription (repression) may completely overshadow the positive effects. The use of HDAC inhibition to chemically dissect a pathway should unmask some important measures of pathway activation that could be overlooked in an undissected system. Indeed, it has been previously observed that the presence of TSA or butyrate indeed uncovered the inducibility of certain reporters that initially had appeared inactive (13, 14).

TSA and butyrate are the most well studied of the HDAC inhibitors for their effects on reporters or integrated genes. Yet, the potential of such general applications of HDAC inhibitors are somewhat controversial, perhaps because the properties of TSA and butyrate in the published reports had been confusing. Various limitations of TSA and butyrate in the applicability to transcriptional assays have been noted in endogenous genes and on the introduction of exogenous sequences. Butyrate and phenylbutyrate have many functions other than inhibiting HDACs; they have been reported to affect the posttranscriptional modification of other genes (15) and the depletion of glutamine (16). There are variable observations that conclude that TSA and other inhibitors do not consistently activate all of the promoters, and such failures of transcriptional facilitation have included the common general-utility promoters CMV and SV40 (17–19). Some of the reported transcriptional actions required a specific small recognition element

(20–22), or the activity of a particular coactivator (23). Furthermore, TSA is not always found to facilitate the detection of positive signal transduction events without interfering with the magnitude of relative transactivation activity (13, 22). It was, therefore, of interest that a more general utility could here be indicated, at least for some HDAC inhibitors within a defined system.

In summary, the identification of scriptaid confirmed the feasibility of compound screening in mammalian cells using this reporter system, in the absence of a formal compound-design effort. Scriptaid is shown to be a novel HDAC inhibitor with robust activity and relatively low toxicity, which suggests a wider utility in transactivation assays and in studies of histone acetylation.

Acknowledgments

We thank Drs. Chris Torrance, Bert Vogelstein, and Ken Kinzler, Johns Hopkins University School of Medicine, for their generous sharing of the compound library. We also thank them and Dr. Jef Boeke also of Johns Hopkins, Johns Hopkins University School of Medicine, for helpful discussions.

References

- Komarov, P. G., Komorova, E. A., Kondratov, R. V., Christov-Tselkov, K., Coon, J. S., Chernov, M. V., and Gudkov, A. V. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* (Washington DC), 285: 1733–1737, 1999.
- Mayer, T. U., Kapoor, T. M., Haggarty, S. J., King, R. W., Schreiber, S. L., and Mitchison, T. J. Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science* (Washington DC), 286: 971–974, 1999.

3. Goggins, M., Shekher, M., Turnacioglu, K., Yeo, C. J., Hruban, R. H., and Kern, S. E. Genetic alterations of the transforming growth factor β receptor genes in pancreatic and biliary adenocarcinomas. *Cancer Res.*, **58**: 5329–5332, 1998.
4. Hahn, S. A., Schutte, M., Hoque, A. T. M. S., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. *DPC4*, a candidate tumor-suppressor gene at 18q21.1. *Science (Washington DC)*, **271**: 350–353, 1996.
5. Whitman, M. Smads and early developmental signaling by the TGF β superfamily. *Genes Dev.*, **12**: 2445–2462, 1998.
6. Dai, J. L., Turnacioglu, K., Schutte, M., Sugar, A. Y., and Kern, S. E. *Dpc4* transcriptional activation and dysfunction in cancer cells. *Cancer Res.*, **58**: 4592–4597, 1998.
7. Finnin, M. S., Donigian, J. R., Cohen, A., Richon, V. M., Rifkind, R. A., Marks, P. A., Breslow, R., and Pavletich, N. P. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Science (Washington DC)*, **401**: 188–193, 1999.
8. Feng, X. H., Zhang, Y., Wu, R. Y., and Deryck, R. The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF- β -induced transcriptional activation. *Genes Dev.*, **12**: 2153–2163, 1998.
9. Janknecht, R., Wells, N. J., and Hunter, T. TGF- β -stimulated cooperation of smad proteins with the coactivators CBP/p300. *Genes Dev.*, **12**: 2114–2119, 1998.
10. Pouponnot, C., Jayaraman, L., and Massague, J. Physical and functional interaction of SMADs and p300/CBP. *J. Biol. Chem.*, **273**: 22865–8, 1998.
11. Richon, V. M., Webb, Y., Merger, R., Sheppard, T., Jursic, B., Ngo, L., Civoli, F., Breslow, R., Rifkind, R. A., and Marks, P. A. Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. *Proc. Natl. Acad. Sci. USA*, **93**: 5705–5708, 1996.
12. Struhal, K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev.*, **12**: 599–606, 1998.
13. Minucci, S., Horn, V., Bhattacharyya, N., Russanova, V., Ogryzko, V. V., Gabriele, L., Howard, B. H., and Ozato, K. A histone deacetylase inhibitor potentiates retinoid receptor action in embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA*, **94**: 11295–11300, 1997.
14. Meng, S., Wu, J. T., Archer, S. Y., and Hodin, R. A. Short-chain fatty acids and thyroid hormone interact in regulating enterocyte gene transcription. *Surgery*, **126**: 293–298, 1999.
15. Kitamura, K., Andoh, A., Inoue, T., Amakata, Y., Hodohara, K., Fujiyama, Y., and Bamba, T. Sodium butyrate blocks (IFN- γ)-induced biosynthesis of MHC class III gene products (complement C4 and factor B) in human fetal intestinal epithelial cells. *Clin. Exp. Immunol.*, **118**: 16–22, 1999.
16. Lea, M. A., and Randolph, V. M. Induction of reporter gene expression by inhibitors of histone deacetylase. *Anticancer Res.*, **18**: 2717–2722, 1998.
17. Huang, Y., Myers, S. J., and Dingledine, R. Transcriptional repression by REST: recruitment of Sin3A and histone deacetylase to neuronal genes. *Nature Neurosci.*, **2**: 867–872, 1999.
18. Zhao, W., Noya, F., Chen, W. Y., Townes, T. M., Chow, L. T., and Broker, T. R. Trichostatin A up-regulates human papillomavirus type 11 upstream regulatory region-E6 promoter activity in undifferentiated primary human keratinocytes. *J. Virol.*, **73**: 5026–5033, 1999.
19. Zabel, M. D., Weiss, J. J., and Weis, J. H. Lymphoid transcription of the murine *CD21* gene is positively regulated by histone acetylation. *J. Immunol.*, **163**: 2697–2703, 1999.
20. Li, S. D., Moy, L., Pittman, N., Shue, G., Aufiero, B., Neufeld, E. J., LeLeiko, N. S., and Walsh, M. J. Transcriptional repression of the cystic fibrosis transmembrane conductance regulator gene, mediated by CCAAT displacement protein/cut homolog, is associated with histone deacetylation. *J. Biol. Chem.*, **274**: 7803–7815, 1999.
21. Xiao, H., Hasegawa, T., and Isobe, K. Both Sp1 and Sp3 are responsible for p21^{WAF1} promoter activity induced by histone deacetylase inhibitor in NIH3T3 cells. *J. Cell. Biochem.*, **73**: 291–302, 1999.
22. Jin, S., and Scotto, K. W. Transcriptional regulation of the *MDR1* gene by histone acetyltransferase and deacetylase is mediated by NF-Y. *Mol. Cell. Biol.*, **18**: 4377–4384, 1998.
23. Sowa, Y., Orita, T., Minamikawa-Hiranabe, S., Mizuno, T., Nomura, H., and Sakai, T. Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor. *Cancer Res.*, **59**: 4266–4270, 1999.

X. Related Proceedings Appendix

There are no related proceedings.